POLYMERIC NUCLEIC ACIDS FROM CITRUS FRUITS AND LEAVES IN VARIOUS STAGES OF GROWTH AND DEVELOPMENT, INCLUDING CHEMICALLY MODIFIED SENESCENCE

By

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INTRODUCTION

Senescence is universal among all living organisms. Every cell of every organism has certain functions during its life span. The cell attains a maximum efficiency at a certain time during its development, then its efficiency begins to decline until death occurs. The controlling factors leading to senescence are incompletely understood.

During senescence, a cell undergoes a variety of physiological and biochemical changes many of which are catabolic in nature. Notable among these changes are those involving protein and nucleic acids. Many investigations (5, 45, 79, 126, 151, 161) have shown that nucleic acids and protein contents of leaves decline drastically during senescence. The ability of a leaf to conserve nucleic acids has been suggested as a major role in the regulation of senescence (79).

In most of these investigations, however, determinations of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) have been made on hydrolysates in which all types of nucleic acids were hydrolyzed with acids or alkalies into soluble nucleotides (42, 106, 146). From such determinations, one cannot tell which types of nucleic acids, if any, have been affected most during senescence. Therefore, it becomes difficult to draw conclusions from such findings. Since various types of nucleic acid have specific functions in cell metabolism, a method of extraction, separation and detection of each type of polymeric nucleotide would help in an understanding of the changes in these molecules during growth and development of citrus fruits and leaves.

Many substances, e.g., growth regulators, have been found to retard senescence of various plant organs. One of these is gibberellic acid (GA) which has been found to delay the disappearance of green color from the rind of many citrus cultivars (28, 31, 34, 65, 85) and of persimmon fruits (78). Other chemicals, e.g., ethylene, are known to accelerate greatly the process of senescence in many fruits, including citrus.

This study was designed to investigate the changes in various types of nucleic acids in the leaves and rind of fruits of Citrus mitis Blanco, calamondin, at various stages of growth and development, including senescence. Attempts were also made to determine the influence of gibberellic acid and ethylene on the polymeric nucleotides of mature fruits and to determine if an altered pattern of nucleic acids was related to an altered pattern of senescence.

REVIEW OF LITERATURE

Plant senescence *per se* has only recently become the subject of investigations (5, 45, 79, 126, 151, 152, 161). Previously, it was usually considered only as a part of the general studies on plant growth and development, with very little attention given to the final stage (113, 144, 173, 174). The increased interest in the study of plant senescence has arisen primarily from the increased attention given to the area of post-harvest physiology of fruits and vegetables. Also, investigations of the physiology and biochemistry of ripening has stimulated more interest in senescence since ripening is one of the most prominent features of fruit senescence (160).

Many of the recent investigations (5, 20, 79, 126, 151, 152, 161) have been conducted on detached leaves and germinating seeds since these organs have an accelerated rate of senescence and they are available and easy to grow and maintain under controlled conditions.

Definition of Senescence

Senescence is the least well defined stage of plant growth and development (82, 99, 125, 126, 132, 170). The difficulty in defining senescence is due mainly to the multiplicity of factors that can bring about senescence and the multiplicity of its manifestations. Varner (170) stated that one reason for the difficulty in defining and studying senescence was the lack of any apparent cause and effect relationship for the metabolic changes associated with it. Nevertheless,

many workers have defined senescence in more or less general terms, but have avoided being specific. It has been described as a problem of unfavorable change in the ability of the cytoplasm to maintain itself (80) and also as the changes that lead to a progressively decreasing power of survival and adjustment (35). Medawar (99) described it as a change accompanying aging which makes an individual more likely to die, and points to the decline in vitality and biological efficiency of an organism with senescence. He admitted that his definition could be critized on many grounds but not for its simplicity.

Leopold (82) defined senescence as a series of changes that leads finally to the death of the organism, but later suggested a more elaborate definition which states that senescence is the deterioration processes which naturally terminate the functional life of an organ or organism (84).

A close examination of these definitions reveals that all are concerned with changes taking place during senescence; yet, in most cases, it is not clear whether these changes bring about senescence or whether they are a manifestation of senescence.

Causes of Senescence

So far no master cause of senescence has been discovered, and the question of "why a cell must eventually die?" posed by Osborne (126), remains virtually unanswered. Some workers have proposed very speculative causes of senescence that, in many cases, are not supported by experimental evidence (170). The proposal by Herzfeld and Klinger (64) that aging was brought about by reduced permeability of cell membrane caused by increased density of protein at the cell

surface which resulted in decreased free exchange across the cell membrane was not supported by any experimental evidence. On the contrary, experiments have shown beyond any doubt that permeability of cell membranes increases, rather than decreases, with senescence (3, 141, 142). Changes in cell permeability have been attributed to changes in osmotic pressure and/or energy relations (3). However, Lansing (80) has demonstrated that calcium accumulates in the cell cortex of a rotifer, a planariun, and a toad with age. He suggested that this peripheral increase in calcium might reduce cell permeability which could interfere with metabolism. By reducing the concentration of calcium in the culture medium of the short-lived rotifer or by experimentally removing the deposited calcium by sodium citrate, Lansing (80) was able to increase the life span of the rotifer by more than 50%.

In 1954, Comfort (35) suggested that senescence was caused by many factors rather than by a single factor, for example, deterioration of cellular and non-cellular molecules and the cumulative effect of previous injuries which were only partially repaired. He did not, however, specify the nature of these injuries but referred to them as "non-specific injuries."

Another hypothesis on the cause of senescence was proposed by Sinex (153) who attributed senescence to molecular changes, as those occurring in proteins and nucleic acids. He based his hypothesis on investigation of the effect of heat treatments on collagen, DNA and hemoglobin. It was found that changes were produced in collagen, but not in DNA or hemoglobin, that were very similar to those produced

in the cell during senescence.

The work of Molisch (104) represents one of the first practical and realistic approaches taken by an investigator toward the subject of senescence in higher plants. Molisch presented the idea that has become known as the 'Death by Starvation' hypothesis. It was suggested that senescence of plants was triggered by the development of flowers and fruits. He supported this suggestion by demonstrating that the life of a plant could be prolonged by the continuous removal of its flowers or fruits before they develop to any great extent. Flower and fruit formation causes the depletion of food reserves from the rest of the plant and this results in its death. Evidence for the migration of food reserves from other parts of the plant to the developing flowers and fruits have been provided both prior to and after Molisch presented his hypothesis. Murneek (113) reported that the rapidly growing seeds of spider flower (Cleome spinosa) were able to monopolize the organic food supply, a process that leads to the production of abortive pistils in the newly developing flowers. Timely removal of fertilized ovaries resulted in the production of other fertile pistils. He suggested that such removal brought about blockage of the drain on food reserves. Vickery (173) estimated that about one-fifth of the plant organic solids and about one-third of the entire plant nitrogen move from all parts of tobacco plants during flowering, particularly from leaves, into the developing seed pods. Watson and Petrie (177) also reported similar findings on tobacco plants during flowering. More recently Mothes et al. (109, 110) showed that labeled amino acids will migrate from one part of

Xanthium leaves to the other under the influence of kinetin. McCollum and Skok (98) also demonstrated that the migration of labeled sugars from leaves into developing tomato fruits was greater into young growing fruits than into mature and ripened fruits. Lockhart and Gottschall (89) found that senescence of the vegetative growth of pea plants could be significantly retarded by detachment of partially filled seed pods. Leopold et al. (81) re-examined the hypothesis of Molisch by conducting experiments on soybean and spinach plants. They reported that the removal of soybean flowers as they were formed resulted in prolonging the life span of the plants by some 60 days. When fruits were allowed to partially develop but were removed before the pods were filled, the life span of the plants was increased by 18 days; whereas, when the pods were allowed to fill but not senesce on the plant, the increase in life span was only 10 days. Defloration of pistillate and staminate spinach plants resulted in similar effects. Conditions that brought about suppression of the development of reproductive organs, such as short photoperiod, resulted in prolonging the life span of vegetative growth. Lockhart and Gottschall (89) found that treatment with gibberellic acid to delay floral development (108) resulted in delaying the senescence of the rest of the plant.

Leopold et al. (81) challenged the death-by-starvation hypothesis on the basis of their findings. They reported that the removal of the inflorescence of staminate spinach plants resulted in maintaining the plants in a rosette condition for a long period of time. It was obvious in such cases that no substantial food reserves were involved in flower formation. Therefore, they suggested that the formation of

reproductive organs triggered a "senescence signal" that was gradually intensified by the progressive development of flowers and fruits (81). This concept was supported by their findings that the effectiveness of deflowering and defruiting treatments in delaying senescence was decreased by increasing the time interval between the beginning of flower development and their detachment. However, the nature of this signal is not yet determined.

Varner (170) suggested that senescence could be caused by the failure of the mitochondria to be replaced or repaired once they have functioned at a given capacity for a given time. This hypothesis was based on the fact that the storage life of fruits and vegetables can be increased by lowering the temperature, a treatment that reduces the rate of metabolism. Thus, this hypothesis may have some validity since the number of mitochondria declines with age (20) and they show signs of internal degeneration in senescing leaves (51, 152). Yet a great deal more needs to be known about mitochondria biogenesis and the relation of mitochondria to growth and development before such a hypothesis can be proved or disproved (170).

Physiological and Biochemical Changes Associated with Senescence

Senescence can be regarded as a final stage in plant development that involves drastic changes in cellular structure and function. The most striking feature of senescence is the hydrolysis of macromolecules. The literature contains many reports of metabolic changes associated with senescence, but the question of whether these changes cause senescence or they themselves are a consequence of senescence has yet to be answered. As Varner (170) suggested, the cause and effect

relationships of these metabolic changes are seldom clear.

1. Changes in Respiratory Activities.

In animal as well as in plant tissues, senescence has been found to be associated with a progressive decline in respiratory rate. Watanabe and Childs (176) reported that the rate of respiration of the polyclad worm declined with age. They also found that sectioning to stimulate regeneration, increased the respiratory rate. Reiner (136) similarly found a sharp decline in the respiratory rate of brain-tissue homogenates of rats with increasing age after maturity. Hover and Gustafson (67) demonstrated a decline in the respiratory rate of wheat, corn, sorghum and oats with age. Yemm (186, 187, 188, 189) reported from his extensive studies on metabolic and respiratory changes in excised barley leaves that the rate of respiration increased sharply after excision then declined to a low level and remained almost constant for 45 hours. Initially, the level of fructose, sucrose and fructosans declined at a rate parallel to the drop in the respiratory rate. The drop in level of these carbohydrate metabolites could account for the carbon loss in respiration during the first 24 hours after detachment. After this, respiration is carried out at the expense of non-carbohydrate metabolites. Similarly, Wood et al. (185) found that the respiratory rate of excised leaves of Kikuyu grass declined as they aged but after showing a characteristic climacteric following detachment. The respiratory rate of many citrus fruits also has been shown to decrease after detachment and the rate to remain at a very low level during senescence(7,8,43). Similar findings were reported on tomato, avocado and pear leaves as they aged (91).

Smillie (157) obtained the lowest values for respiration with primary pea leaves as they senesce. Geronimo and Beevers (51) demonstrated that the rate of respiration of pea leaves declined by about 40 to 60% during the early stages of senescence. Mitochondrial fractions isolated from senescent pea leaves showed also a decline of between 40 and 70% in the rate of utilization of acetate-C^{14}. This closely paralleled the drop in the respiratory activity. Furthermore, there was a decline in the phosphate/oxygen uptake ratio (P/O) and in the activity of the enzymes involved in the electron transport system (51).

Electron microscopic examination of mitochondria of young and senescent pea leaves have shown that as the leaf ages the mitochondria exhibited interal degeneration and became fewer in number in the cell (51). Shaw et al. (152) reported that mitochondria in senescent mesophyll cells of wheat leaves began to swell and their cristae began to degenerate 3 days after detachment. These observations are logical since high respiratory activities, including oxidative phosphorylation, has been shown to be associated with well organized mitochondria that have a well developed cristae (90). It has been suggested by Geronimo and Beevers (51) that the drop in respiratory activities which accompanies senescence of pea leaves and possibly senescence in general, may be brought about by the decreased efficiency of mitochondria to utilize energy-rich substrates. This could be brought about by the disorganization of the interal structure of the mitochondria.

Treatment of mitochondrial preparations from root cells of corn with low concentrations of ribonuclease resulted in an increased O_2

uptake and a decreased P/O ratio (60, 61). Higher concentrations resulted in a decline in O_2 uptake and uncoupled oxidative phosphorylation. It is suggested that a change of this nature could be taking place during natural senescence with the concommitant decline in respiration rate and uncoupling of oxidative phosphorylation. This is supported by Cherry's findings of increased ribonuclease activity in aging root cells of corn (20), and by those of Srivastava and Ware (161) of increased ribonuclease and deoxyribonuclease activities in excised barley leaves. Moreover, an increase in the activity of ribonuclease could lead to a disruption of cellular membranes. Sacher (141) examined leaf sections of Rhoeo and Mesembryanthemum as they aged, and found that an increase in plasmolysis and in the rate of leakage of cellular content closely paralleled the decline in respiratory rate. The rate of leakage could be slowed by floating the leaf sections on an aqueous solution of indole-3-acetic acid (IAA) or naphthaleneacetic acid (NAA) instead of just water. Therefore, it was suggested that auxins were necessary for maintaining functional membranes (141).

Baur and Workman (3) similarily showed that ion leakage increased progressively as the respiratory rate of post-climacteric banana fruits begins to decline. They attributed this to lost membrane integrity brought about by either the increase in osmotic pressure caused by starch to sugar transformations, or by changes in an energy relation caused by uncoupling of the oxidative phosphorylation. It is interesting to note that Sacher (143) measured free space in banana tissue, using labeled mannitol, sucrose and fructose

and found that the increase in permeability precedes the respiratory climacteric by some 36 to 40 hours.

Hanson (61) suggested that divalent ions may be involved in ion accumulation, solute retention, and oxidative phosphorylation of mitochondria preparations from corn and soybean roots by protecting the mitochondrial RNA against the action of ribonuclease. A similar role has been assigned to zinc in citrus leaves (70). Monovalent ions have been found to cause the degradation of mitochondrial RNA, an increase in permeability and a loss of oxidative phosphorylation capacities (161).

Recently Lin and Key (87) reported that polyribosomes from soybean roots lost their integrity when the roots were maintained under anaerobic conditions. Geronimo and Beevers (51) and Shaw et al. (151) demonstrated that a reduction in respiratory activity was associated with an internal degeneration of mitochondria and the disappearance of ribosomal particles. These results provide additional support for the hypothesis which suggests that senescence may be caused by the deterioration of mitochondria with age, and failure of the cell to repair or regenerate its mitochondrial particles (170).

2. Changes in Photosynthetic Activities

The assimilatory capacity of plants has been found to be altered during senescence. Singh and Lal (154) reported that the photosynthetic activity of wheat, linseed and sugar cane leaves, measured in terms of dry matter, increased as the leaf expanded, and reached a maximum value as the leaf reached its maximum size. Then it declined with age. Smillie (157) obtained similar results with

primary leaves of Pisum sativum and photosynthesis reached its lowest level just before the leaf abscised. Sestak and Catsky (149) measured photosynthetic activity of tobacco leaves of increasing age on the same plant and found it to decline with age. With citrus fruits, photosynthetic activity has been found to decline sharply when the rind begins to yellow (4), as a result of chlorophyll destruction. Citrus leaves, however, have a relatively long life span and may remain photosynthetically active for about two years (71, 137, 141, 147). In bean leaves, photosynthesis declined sharply as the leaf senesced to the extent that it began to decrease in dry weight (37).

Chemical treatments with kinetin (125, 126), IAA (167) and 2,4-dichlorophenoxyacetic acid (2,4-D) (124) have been found to delay the decline of photosynthetic activity and to prevent losses in dry weight, chlorophyll and protein content of leaves. Turner and Bidwell (167) showed IAA to stimulate the photosynthetic rate of bean leaves and found no great difference in the photosynthetic pattern of detached or attached leaves. Osborne (124) suggested that auxins may be controlling some factors in protein synthesis. Losses in chlorophyll during senescence have always been found to be associated with losses in protein when both were measured (126, 138, 151, 161). It has also been reported that the chloroplasts contained a major portion of the cytoplasmic protein (25,183), and it was suggested that the losses in chlorophyll and protein may be structurally related (84). More conclusively, Pogo and Pogo (133) found that inhibition of protein synthesis by chloramphenicol was associated with an inhibition of chlorophyll formation in Euglena. This may be considered as evidence of a maintenance relationship between protein and chlorophyll in the chloroplasts.

3. Changes in Chlorophyll Content

One of the most prominent features of senescence in higher plants, is the destruction of chlorophyll, bringing about the loss of green color of healthy plants and the decline of photosynthetic activities. Leopold (82,84) gave a typical example of this dramatic feature of senescence by the massive death of thousands of wheat and corn plants in a field that follows the apparent loss of the green pigments from the leaves and the stems. A similar massive destruction of chlorophyll takes place in the leaves of decideous trees in the fall. Chlorophyll destruction takes place in senescing leaves whether attached to the plant or after excision; however, the rate of yellowing is accelerated by excision (189). The accelerated rate with which senescence proceeds in detached organs has been related to loss of root factors for senescence symptoms will proceed rapidly on detached bean and ivy leaves; but, if roots form on the petiole, the leaves will maintain their initial levels of chlorophyll and protein, and will remain photosynthetically active (54).

Many investigations have been conducted on the relation of the chlorophyll content of plant organs to senescence, including Xanthium leaves (125, 126, 138), wheat and barley leaves (129, 151, 161), bean leaves (37), head lettuce (88), Prunus leaves (123), soybean leaves (79), pea leaves (156), broccoli (40), Nasturtium leaves (5), Taraxacum leaves (45), and many citrus fruits (29, 30, 31, 85). In these studies, chlorophyll content has been shown to decline as the plant organ senesces.

4. Changes in Protein and Nucleic Acids

All living cells undergoing senescence exhibit a variety of metabolic changes, the outcome of which is, seemingly, increased catabolic and decreased anabolic activities. Among these changes are those involving protein and nucleic acid polymers, resulting in a pronounced alteration in these vital cell components. Germinating seeds and excised leaves provide an excellent experimental material for studies on protein and nucleic acid changes during senescence. In the course of seed germination, cotyledons are depleted of their food reserves in a matter of a few days (20, 121), and leaf excision speeds up the rate of degradative processes associated with senescence (131, 189). Following detachment, a leaf undergoes a decline in protein nitrogen and an increase in amide and amino nitrogen (132, 179, 186, 187, 188). As senescence progresses, protein declines further and ammonia is released due to the oxidation of amino acids and amides (132, 179, 186, 187, 188). Wood and Cruickshank (184) showed that the loss in protein nitrogen could be accounted for by hydrolysis of protein to amino acids and the subsequent oxidation of amino acids to keto acids and ammonia. Loss of protein is not confined to cytoplasmic proteins but also included chloroplastic proteins (25, 188). The degradation of both fractions of proteins takes place concurrently (25). Plaisted (132) reported that protein content of attached senescing maple leaves declined sharply at approximately 50 days before natural abscission. There was first an increase in all amino acids, except aspartate, glutamate and alanine accompanying the decline in protein followed by the production and release of ammonia. It was suggested that the

decline in these three amino acids may have been due to the fact that they were the first to be formed in photosynthesis and that the decline in photosynthetic activity with age and their use as precursors to other amino acids leads to their decline. Weinstein (179, 180) also reported that the protein content of the petals of cut 'Better Time' roses declined by as much as 40% in 96 hours, resulting in a build-up in amino acids and ammonia. Incorporation of $C^{14}O_2$ into petal protein declined with age (180). Richmond and Lang (138) found that the protein content of Xanthium leaf discs had declined to 40% of their initial level after 20 days of floating on water. Similar findings have been reported for wheat and barley leaves (151, 161), Xanthium leaves (126), Prunus leaves (123), tobacco leaves (109), Nasturtium leaves (5) and Taraxacum leaves (45).

Nucleic acids, especially RNA, also undergo a drastic decline during senescence. Smillie and Krotkov (155, 156) demonstrated that the RNA content declined in senescing pea leaves. The decrease was associated with a decline in both oxidative and photosynthetic phosphorylation activities. Osborne (126) reported that both the RNA and DNA contents of Xanthium leaves declined during senescence. Similar findings have been reported for cereal leaves (151, 161), Nasturtium leaves (5), Taraxacum leaves (45), and leaves of deciduous trees (49, 72).

Proteins and nucleic acids have been studied in germinating seeds by many workers (20, 118, 119, 120, 122). Cherry (20) found that the RNA content of peanut cotyledons tripled in 8 days and then decreased, while the DNA content doubled by the 10th day of germination

and then declined. The decline in nucleic acids in the cotyledons was found to be associated with a decline in the P/O ratio and disorganization of the internal structure of the mitochondria (20). In germinating bean seeds, RNA declines in the cotyledons while it increases in the radicle, plumule and hypocotyl (122). At least part of this RNA was found to originate in the cotyledons (118, 119, 120, 121). Excised cotyledons retained almost all of its initial RNA while those attached lost almost 50% of their RNA after a 48-hour incubation period (118).

Using an electrophoretic technique of analysis, Oota and Takata (119) found that the mobility of the cotyledonary nucleoprotein increased with the progress of germination. The increase in mobility was attributed to dissociation of RNA from protein. Then the free RNA, which they termed "transportable RNA," migrated to the growing tissues of the seedling.

The loss of protein during senescence is apparently caused by both increased proteolysis and impaired synthesis. Impairment of the system or systems involved in protein synthesis seems to contribute much to the decline in protein content (175). Since nucleic acids and protein metabolisms are very closely related (66), disturbances in nucleic acid metabolism could result in disruption of protein synthesis. The ribosomal particles, particularly the ones associated with the endoplasmic reticulum, are the site on which amino acids are assembled into proteins (41). If the number of these sites is reduced, a decline in the rate of protein synthesis will follow.

Pirie (130) found a 30-fold decrease in the extractable nucleoproteins from tobacco leaves with age. Lund et al. (90) found that

the density of microsomal granules in cells of corn roots declined with age. Shaw and Manocha (152) reported that a general disorganization of cellular organelles took place in senescing wheat leaves. Cytological studies revealed that most of the nuclei had disintigrated after 6 to 8 days from excision of wheat leaves and that the ribosomes had disappeared completely after 4 to 5 days. Breuer and Florini (11) reported that the polyribosomal content of microsomal fractions isolated from the thigh muscle of rats declined with age of the animal. Wood and Bradbeer (182) reported that ribosomal-RNA-(r-RNA) and the DNA-RNA complex in cotyledons of hazel seed declined sharply in the latter stages of germination. Sturani and Cocucci (164) noted that the polyribosomes and ribosomes in endosperms of castor bean seeds disappeared as the seed matured. Richmond and Biale (139) studied the nucleic acids and their labeling behavior with P^{32} with preclimacteric, climacteric and post-climacteric avocado fruits. They reported a shift in labeling from r-RNA to soluble-RNA (s-RNA) as the tissue aged. Also, Cherry et al. (23) reported that r-RNA declined and s-RNA increased in peanut cotyledons with age. This may indicate that at least part of the decline in protein content of senescing plant organs is brought about by impaired synthesis. This concept is supported by work on senescing Xanthium leaves (126). Osborne (126) found that the ability of leaf discs to incorporate C^{14}-leucine and C^{14}-orotic acid into protein and nucleic acids declined with age. Furthermore, Breurer and Florini (11) found that the capacity of ribosomes isolated from rat muscles to incorporate amino acids into protein to decrease with aging of the animal. Racusen

and Arnoff (135) also showed that the ability of tissues of bean leaves to incorporate labeled amino acids into protein decreased as the time between excision and the feeding of $C^{14}O_2$ increased. Similarly, Sacher (143) reported a reduction in the incorporation of C^{14}-labeled leucine and phenylalanine in senescing banana tissue. According to Rogers (140), excised plant organs are still capable of synthesizing amino acids. Plaisted (132) and Yemm (188) found that amino acids accumulated in senescing cells due to excessive protein breakdown. Therefore, it seems possible that the reduction in protein content during senescence could be partially caused by impaired protein synthesis from an upset in nucleic acid metabolism, particularly the components needed for the polymerization of amino acids to proteins.

The Role of Nucleic Acid Metabolism In Regulation of Physiological Processes

The relationships between nucleic acids and protein metabolism have been well established (66). The widely accepted concept suggests that the genetic material, which consists mainly of DNA, directs protein synthesis on the surface of the ribosomes by sending specific molecules of messenger-RNA (m-RNA) that carry the genetic information needed for the synthesis of a particular type of protein molecules. The synthesis of various types of proteins, including enzymes, would then bring about the various physiological and biochemical changes.

The current trend in physiological studies is to relate nucleic acids and protein metabolism to the various physiological processes of plants. Smillie and Krotkov (155) found a close parallelism between RNA level of pea leaves and its oxidative and photosynthetic

phosphorylation activities. They suggested that RNA level could be used as an indicator of the metabolic activity of leaves at various stages of development. Biswas and Sen (9) found that the auxin IAA at physiological levels stimulated the incorporation of P^{32} into RNA of Avena coleoptiles while higher concentrations inhibited both growth and P^{32} incorporation into RNA. Similarly Key and Hanson (73) demonstrated that the application of 2,4-D in concentrations of 100-800 ppm to sections of soybean hypocotyl resulted in large increases in nucleic acids and soluble nucleotides. Furthermore, Shannon et al. (150) found high concentrations of 2,4-D to inhibit ribonuclease activity. Using C^{14}-labeled adenosine diphosphate they showed that 2,4-D induced qualitative changes in RNA; changes which could not be detected by quantitative measurement. West (181) also reported that corn seedlings grown under water stress showed an increase in an RNA of an abnormal base composition. It was suggested that 2,4-D may induce the synthesis of a particular type of metabolically active RNA that mediates certain influences of auxin (73). Nooden and Thimann (115) presented evidence that the action of auxin on cell elongation was mediated by a nucleic acid system that controlled the synthesis of an essential protein. Their conclusion was based on experiments in which they used chloramphenicol, puromycin, and actinomycin D to inhibit protein, DNA and RNA synthesis, respectively.

All three types of inhibitors were found to inhibit cellular elongation of Avena coleopile sections induced by auxin under certain conditions. In 1964, Key (74) and Key and Shannon (76), using C^{14}-nucleotides found also that auxin-induced elongation of cells was

associated with RNA synthesis. They presented evidence to show a net transfer of C^{14}-RNA from the nucleus to the ribosomes. They also found that actinomycin D inhibited RNA synthesis by 80-90%. Since actinomycin D has been found to inhibit RNA synthesis by binding to the DNA template which controls RNA synthesis (52, 62), Key and Shannon (76) suggested that new RNA of soybean hypocotyl must be synthesized on a DNA template. Further evidence was presented by Key and Ingle (75) who showed that the growth of soybean hypocotyls, radish cotyledons and corn mesocotyl tissues required the synthesis of a DNA-like RNA similar to that obtained from bacterial cells (62), and peanut cotyledons (21). The fact that actinomycin D inhibited synthesis of DNA-like RNA (52, 62, 68, 100) and growth (115), whereas 5-fluorouracil inhibited the synthesis of s-RNA and r-RNA without inhibiting growth (17, 75), led Key and Ingle (75) to suggest that cell enlargement was mediated by a m-RNA under the control of a specific genome.

Molotkouskii and Moryakova (105) have shown a relation in etiolated bean seedlings between RNA and protein synthesis on one hand and chloroplast formation and chlorophyll synthesis on the other. It was found that the inhibition of protein synthesis by chloramphenicol and RNA synthesis by thiouracil, azauracil or maleic acid hydrazine, resulted in inhibition of chloroplast formation and chlorophyll biogenesis. Reversal of the inhibitory effects of thiouracil by the administration of uracil led Molotkouskii and Moryakova (105) to suggest that light-induced formation of chloroplasts and chlorophyll was directed by the synthesis of a specific RNA. This in turn controlled the formation of chloroplastic protein necessary for chlorophyll synthesis.

Another example of the relation between nucleic acid metabolism and growth and development is provided by Tuan and Bonner (166). They reported that actinomycin D inhibits RNA synthesis in active potato tuber buds; whereas it has no effect on RNA synthesis in dormant buds. They suggested that the genetic material in dormant buds was repressed and that the breaking of dormancy was a result of allowing gene expression.

The wide range of physiological and biochemical influence of animal hormones and plant growth regulators is well documented, but the mechanisms by which they act is still a mystery in most cases. Recently much evidence has been presented that indicate that hormonal action is mediated through the genes, and in certain cases, results in the activation of certain ones (38). This activation induces the synthesis of certain types of m-RNA that directs the synthesis of certain types of proteins, including catalytic ones. In turn, these enzymes regulate the metabolic processes by having certain inherent specificities. Evidence for such a concept has come mainly from studies on animal tissues; however, there are a few studies on plants (46, 105, 166, 171, 172).

The synthesis of nucleic acids is stimulated by many hormones and auxins (50, 53, 59, 69, 73, 75, 76, 86, 115,134).Kinetin-induced cell division involves enhanced synthesis of nucleic acids (57, 102, 163). Similarly, auxin-induced enlargement of cells requires RNA, DNA-RNA and protein synthesis (74, 75, 115).

Gibberellic acid increased the activity of α-amylase in the barley endosperm (127, 128). Varner and Chandra (171, 172), and

Flinter and Varner (46) demonstrated that the increased enzyme activity induced by GA was caused by de novo synthesis of α-amlyase. Since this mechanism was susceptible to inhibition by actinomycin D, it was suggested that the synthesis of a DNA-dependent RNA was involved in the action of GA on α-amylase synthesis. Van Overbeek (169) speculated that GA may act by uncovering specific genes resulting in the synthesis of a specific m-RNA that induced the formation of specific enzymes.

The interaction between certain hormones and nucleic acids, or their products, has been demonstrated in both animals and plants. Scott (148) reported an interaction in vitro between testosterone and purine-containing substances. A similar interaction between testosterone and a number of coenzymes has also been reported (112). Tso and Lu (165) also found testosterone to bind to calf thymus histones and denatured DNA. Bendana et al. (6) reported that C^{14}-labeled IAA was incorporated into RNA of pea stem sections. Srivastava (162) reported the incorporation of intact C^{14}-kinetin into soluble and ribosomal RNA, and Fox (47, 48) reported that N^6-benzyladenine was incorporated into s-RNA of tobacco tissue culture.

There are still many problems that need to be solved, chiefly how to detect specific m-RNA polymers. As Cherry (24) indicated, there are probably hundreds of m-RNA polymers in each cell and at low concentrations. This is a new challenge for biochemists and physiologists.

Chemical Modification of Senescence

So far, it has not been possible to completely prevent senescence with any treatment. In plants, however, the degradative processes associated with senescence can be delayed by certain chemicals. Complete inhibition of senescence is an objective that has not yet been accomplished, except in cell and tissue cultures where cells can live indefinitely as long as they are kept dividing and the products of catabolism are removed and fresh nutrients are supplemented (145, 163).

Certain chemicals have been found to delay senescence in plants, notable among them are the kinins. Kinetin (6-furfural aminopurine) was the first kinin to be discovered (102). It was found to stimulate cell division (36, 56, 57, 102, 163), delay senescence of localized areas of tobacco leaves (109, 110, 111) and induce the migration of various metabolites from non-treated to treated areas. Other investigations (79, 126, 151, 152, 161) demonstrate that kinetin will prolong the life of excised leaves. Retardation of senescence was found to be associated with a higher level of chlorophyll, protein, RNA and DNA in the tissue. It has also been reported (161) that kinetin-treated barley leaves have a lower ribonuclease and deoxyribonuclease activities compared to non-treated leaves, and that the rate of P^{32} incorporated into RNA and DNA fractions in the treated detached leaves was maintained at a level close to that of intact leaves. The exact mechanism by which kinetin acts in delaying senescence is not fully understood. It has been suggested to act by maintaining the level of nucleic acid synthesis and by preventing the breakdown of chlorophyll, proteins and nucleic acids (79, 126, 151, 161).

Other chemicals that slightly delay senescence include 2,4-D (123), benzamidazole (129), N^6-benzyladenine (39, 83, 88, 97), N^6-benzylamino purine (40), 2-chloroethyltrimethylammonium chloride, and N, N-dimethylaminosuccinamic acid (58). Also, gibberellic acid delays senescence of excised Nasturtium and Taraxacum leaves (5, 45), and certain citrus fruits (26, 27, 28, 29, 30, 31, 33, 65, 86). In contrast, other workers (58, 107) report that GA hastened senescence of bean and citrus leaves. It would seem that different plants or plant organs may respond differently. Flinter and Osborne (46) examined the effect of GA on the senescence of leaves of 18 species and found only Taraxacum leaves to respond to GA treatment by having their senescence delayed. Leaf discs treated with GA maintained higher levels of chlorophyll, protein and nucleic acids than nontreated discs.

Recently, Beevers (5) found GA to have a similar effect on Nasturtium leaves. He suggested that various plants may respond differently to different growth regulators due to an imbalance between hormonal factors that may result in gibberellin being limited in one plant, kinins in another, etc. This idea is supported by the following observations. Osborne (123) reported that senescence of Prunus leaves could be delayed by treatment with 2, 4-D, while kinetin failed to show any retardation (125). On the other hand, kinetin was effective in delaying senescence of Xanthium leaves (126), while the effect of IAA was erratic (138).

The action of GA in delaying the regreening of the rind of nearly mature citrus fruits has been well documented (26, 27, 28, 29, 30, 31, 33, 34, 65, 86). These studies have been conducted on 'Washington' navel

and 'Valencia' oranges, grapefruit, 'Lisbon' lemon, and 'Clementine' mandarin. The action of GA varied from one variety of citrus to the other. With 'Valencia' oranges it stimulated regreening of the rind of ripened fruits (30). This was also the case with grapefruit (31); however, regreening has not been observed in 'Washington' navel orange (29); yet 'Washington' navel fruits treated with GA do not degreen as rapidly as non-treated fruits (85). Lewis and Coggins (85), found that the action of GA and light on chlorophyll content of 'Washington' navel' oranges was additive. GA-treated fruits under normal light conditions had a higher chlorophyll content than those under low light intensities, indicating that chlorophyll was still being synthesized during the change from green to orange (85). Such findings, along with those on the stimulation of regreening of the rind of fully ripened 'Valencia' orange (30) and grapefruit (31), led Lewis and Coggins (85) to hypothesize that GA stimulates chlorophyll synthesis. The action of GA in delaying color-break of mature fruits of citrus indicates that it delays rind senescence (29). In examining the effect of GA on other characteristics of the rind of citrus fruits, Coggins and Eaks (33) found it to reduce the incidence of rind staining, sticky rind and water spot in 'Washington' navel orange. It also decreased the susceptibility of the rind to rupture under pressure (33, 34).

In contrast to the action of kinetin and GA in retarding senescence, ethylene enhances the reactions associated with senescence. It is used commercially to degreen citrus (55) and hasten the ripening of banana. It also causes leaf epinasty and has been found effective in breaking seed and bud dormancy (14). The mode of action of ethylene

is not yet understood, nor is it known how or where in the cell it is produced. A discussion of ethylene biogenesis and action can be found in reviews by Burg (14), Burg and Burg (15) and by Spencer (160).

MATERIALS AND METHODS

Plant Materials

A. Calamondins

Two-year old calamondin (Citrus mitis Blanco) cutting-grown trees in one-gallon containers (Fig. 1) were obtained from a commercial nursery. The trees were kept in a greenhouse during the winter months and outside during the spring and summer months. Each series of tests was completed while the trees were either inside or outside the greenhouse to eliminate the effect of variable environmental conditions.

1. Sampling of fruits. Five stages of fruit growth and development were arbitrarily selected on the basis of fruit diameter (Fig. 2):

Stage I:	0.5 - 1.0 cm diameter immature fruits
Stage II:	1.0 - 1.5 cm diameter immature fruits
Stage III:	1.5 - 2.0 cm diameter immature fruits
Stage IV:	2.0 - 2.5 cm diameter immature fruits
Stage V:	2.0 - 3.0 cm diameter ripe fruits

Fruits in the first three stages of development were dark green and were considered immature since they had the capacity for further growth. Those in the fourth stage were light green in color and had attained or were close to attaining their maximum size. Fruits in the fifth stage were completely orange in color and fully ripened.

Fig. 1. Cutting-grown calamondin tree (Citrus mitis Blanco).

Fig. 2. Calamondin fruits at various stages of growth and development.

Composite samples from 10 trees were used in each experiment. The fruits were harvested, washed, peeled and a 10 g sample of the rind was used for the analysis of nucleic acids.

2. Sampling of leaves. Leaves at three distinct stages of development (Fig. 3) were used for the nucleic acid analyses, young expanding leaves, 2-3 cm long and light green in color; fully expanded and mature leaves, 7-8 cm long with a leathery texture and dark green color; and senescing leaves which had the same length as the fully expanded leaves but began to yellow. Two 10 g samples of leaves of each stage were extracted for nucleic acids.

B. Other citrus cultivars.

'Parson Brown', 'Valencia' and 'Pineapple' orange fruits were used in preliminary experiments on the influence of GA on the chlorophyll content of citrus fruits.

Treatment to Alter Senescence

A. Ethylene treatments.

The plants were placed in a small commercial degreening room for 24 hours. Ethylene was delivered to the chamber at a continuous rate of about 150 ppm. The temperature of the chamber was 28±2C and the relative humidity was 85±5%.

For the first series of tests, two trees were used. Each tree was bearing green mature fruits. One tree was subjected to ethylene, the other was used as a control. Samples of mature green fruits, and mature leaves were taken prior to the commencement of the 24-hour period for analysis. Immediately after the 24-hour period, the trees

Fig. 3. Calamondin leaves at three different stages of growth and development.

were removed from the degreening room and samples were taken of the treated and non-treated plant. In addition to the leaf and fruit samples for immediate analysis, fruits were collected and stored in polyethylene bags for analysis at various time intervals during senescence. Storage conditions were 20±2C with continuous illumination of approximately 300 ft-c.

B. Gibberillic Acid treatments.

1. Calamondins. Tests were conducted on excised, fully expanded, mature fruits. A sample of 60 fruits was selected from ten trees for uniformity of color and size. Average diameter of the fruits was 2.8 cm. Twelve fruits were used for nucleic acids and chlorophyll analyses immediately after excision to determine the initial level of these components. Twenty-four fruits were treated with a 500 ppm GA solution by submerging the fruits for 1 minute and then allowing them to dry in an air-conditioned room at about 20±2C. Groups of 12 fruits were stored separately in polyethylene bags at 20±2C with 300 ft-c illumination. Fruits were used for P^{32} incorporation studies and for nucleic acids and chlorophyll analysis after 14 and 28 days from the beginning of the treatment.

2. Three sweet orange cultivars of Citrus sinensis. Four experiments were conducted on detached fruits, since temperature is known to have a critical effect on color development in citrus fruits (16, 44, 55).

a. 'Parson Brown' fruits. Mature green 'Parson Brown' fruits were harvested from 19-year old trees on sour-orange rootstock. Fruits were selected for uniformity of size and color. Samples consisted of

6 fruits per treatment with three replications. A 6-fruit sample was used to determine the initial chlorophyll content of the rind. Six 6-fruit samples constituting two treatments were submerged for 1 minute in a 500 ppm GA solution. Three of these samples were stored with a control in a chamber with a light intensity of 2 ft-c for 12 hours a day. Light was supplied with incandescent lamps in both cases. Temperature in the two chambers was 25±2C. A 6-fruit sample was withdrawn from each treatment at 10-day intervals for chlorophyll determination.

b. 'Valencia' orange fruits. Mature green 'Valencia' orange fruits were harvested from 11-year old trees on rough lemon rootstock. Fruits were selected for uniformity of size and color. Samples consisted of 10 fruits each. Three samples were used per treatment. A circle, 2 cm in diameter was marked on the equatorial plane of each fruit. Color within the circle was determined as reflectance value at 675 mμ using a Bausch & Lomb "Spectromic 20" spectrophotometer equipped with a reflectance unit. Six 10-fruit samples, constituting two treatments, were submerged in a 500 ppm GA solution for 1 minute. One group was stored in the dark while the other was stored under a light intensity of 300 ft-c supplied by a combination of incandescent and fluorescent lamps. Temperature was maintained at 24±2C. Two other groups were stored as controls. Color change was measured weekly for 5 weeks on the same circle.

Tests of Various Gibberellic Acid Concentrations On Color Change in the Rind of 'Valencia' Orange Fruits:

'Valencia' fruits used in these tests were sampled as described under part b. Color determinations were made at weekly intervals for a period of 5 weeks using the "Spectronic 20" spectrophotometer.

Treatments consisted of dipping the 30 fruits that constituted each treatment for 1 minute in 0, 10, 30, 60, 100, 500 or 1000 ppm GA solutions. Fruits were stored in polyethylene bags and cardboard boxes at 24±2C.

Tests of Gibberellic Acid on Regreening of the Rind In 'Parson Brown' and 'Pineapple' Orange Fruits:

'Parson Brown' and 'Pineapple' orange fruits were harvested from 26- and 39-year old trees respectively, all on rough lemon rootstock. Fruits were completely orange in color except for occasional light green spots that persisted in the rind of 'Parson Brown' fruits. Three 10-fruit samples were treated with 500 ppm GA solution for 1 minute and stored under light intensity of 300 ft-c. Color change, within a premarked, randomly selected spot on the rind, was followed weekly for 5 weeks by measuring the reflectance at 675 mμ.

Analytical Methods

A. Analysis of rind tissue for chlorophyll content.

Chlorophylls were extracted from the rind of 'Parson Brown' orange and calamondin fruits according to a method described by Smith and Benitez (158) and as used on citrus fruits by Coggins and Lewis (30). Three samples of eight rind discs, 15 mm in diameter, were selected randomly from around the equatorial plane of the fruits. The

rind discs were ground in 80 ml of acetone for 6 minutes at maximum speed and 2 minutes at a medium speed in a Servall Omni-mixer equipped with a rheostat. The mixture was then transferred to a 200 ml Erlenmeyer flask with 40 ml of diethylether and stored overnight in the dark at 4±2C. The mixture was then filtered and the residue was washed with about 50 ml of diethylether until it was free of chlorophyll. The filterate was washed 3 times with a 100 ml portion of glass-distilled water in a separatory funnel. The aqueous layer was discarded after every wash. The ether extract of chlorophyll was reduced to a 15 ml volume under a stream of nitrogen and stored overnight in the dark at 4C. The absorption of the extracted pigments was measured at 660 and 642.5mμ with a Beckman DU spectrophotometer and the total chlorophyll was calculated by the formula given by Smith and Benitez (158) which is as follows:

$$\text{Total Chlorophyll (mg/l)} = 7.12 \log \left(\frac{I_o}{I}\right)_{660} + 16.8 \log \left(\frac{I_o}{I}\right)_{642.5}$$

B. Analysis of leaf and rind tissues for polymeric nucleic acids.

The RNA extraction method utilized in this work is a modified version of one that was first introduced by Kirby in 1959 (77) and later modified by Cherry (21) and Cherry et al. (23). For this study, the method was modified to extract nucleic acids from the rind of citrus fruits mainly by changing the grinding procedure and by increasing the amounts of reagents used. Modifications are as follows: A 10 g sample of rind tissue was ground in an Omni-mixer, keeping the grinding container at about 3C in a mixture of the following reagents:

a. 45 ml of 81% phenol saturated with a mixture of 0.01 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.06 M KCl,

b. 40 ml of 0.01 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.06 M KCl,

c. 10 ml of 5.5% sodium lauryl sulfate,

d. 5 ml of 40 mg/ml bentonite suspension in acetate buffer, pH 6.

The standard bentonite suspension was prepared by the method described by Brownhill et al. (13). Briefly, the coarse and the very fine particles were eliminated by homogenization and centrifigation using an Omni-mixer and a Servall centrifuge, respectively. The medium-sized particles were successively washed with a pH 6 acetate buffer and finally were suspended in the same buffer when the absorbancy value at 260 mμ of the supernatant dropped below 0.7. The concentration of the final suspension was adjusted to 40 mg/ml with acetate buffer, pH 6.

The sample was ground in an Omni-mixer for 5 minutes at full speed, 1 minute at a low speed and then 2 minutes at full speed. The mixture was centrifuged at 20,000 x g and 0 C for 10 minutes. The aqueous layer was transferred with a syringe to a beaker containing 5 ml of the bentonite suspension and the mixture was maintained at 4 C. The phenol layer was removed and discarded. The residue and the interphase layer were transferred to a pre-chilled mortar with 10 ml of 5.5% sodium lauryl sulfate. The residue was ground with a mortar and pestle for about 15 minutes to obtain a homogenate without coarse particles. Then, the homogenate was transferred to polypropylene centrifuge tubes with 45 ml of cold phenol and 40 ml of reagent b. It was centrifuged at 20,000 x g and 0 C for 10 minutes. The aqueous layer was combined with that obtained in the first extraction and an equal volume of cold phenol was added to it and the mixture was left at

4 C for 15 minutes with occasional stirring. Then it was centrifuged at 20,000 x g and 0 C for 10 minutes, and the aqueous layer was transferred to a chilled beaker containing 3 ml of bentonite suspension. Again the aqueous portion was mixed with an equal volume of cold phenol. After 15 minutes of occasional shaking at 4 C, the mixture was centrifuged at 20,000 x g and 0 C for 10 minutes. The alcohol precipitable portion of the aqueous layers were obtained by adding enough cold absolute ethanol to make it 66% ethanol after making the aqueous fraction 2% with respect to potassium acetate. The precipitating reaction was allowed to occur at 4 C for 4 hours. The mixture was then centrifuged at 30,000 x g and 0 C for 20 minutes. The pellets were dissolved in approximately 20 ml of cold 0.05 M sodium phosphate buffer, pH 6.7.

The solution was again subjected to the same procedure of alcohol precipitation and centrifugation. The pellet was redissolved in approximately 20 ml of cold 0.05 M sodium phosphate buffer, pH 6.7, and dialyzed for 48 hours against the same buffer used to dissolve the pellet. During dialysis the buffer was changed three times.

C. Chromatography of nucleic acids on methylated albumin-Kieslguhr (MAK) columns.

The MAK method of chromatography of nucleic acids was developed by Mandell and Hershey (93) and was successfully applied to nucleic acid extracts of various plant materials (12, 21, 23, 24, 25, 75).

1. Preparation of methylated-albumin (MA). Two and one-half grams of bovine-serum albumin (fraction V) were suspended in 250 ml of absolute methanol. After adding 4.2 ml of concentrated HCl, the mixture

was incubated in the dark for 5 days at 37 C. Then the mixture was centrifuged at 10,000 x g for 5 minutes and the MA pellet was separated, resuspended in absolute methanol and centrifuged again at 10,000 x g. This washing procedure was repreated two additional times with absolute methanol and two times with diethylether. The MA pellet obtained after the last centrifugation was then dried in a hood and ground to a fine powder with a mortar and pestle. A 1% MA solution was made up and the rest of the powder was stored in a vacuum desiccator over KOH in a freezer until needed. The aqueous solution was used immediately or stored at 4 C for no longer than 3 weeks.

2. Preparation of methylated albumin-coated Kieslguhl (MAK). Twenty grams of Kieslguhr was suspended in 100 ml of 0.1 M NaCl in 0.05 M sodium phosphate buffer, pH 6.7, boiled, and cooled in an ice bath. To the cold suspension, 5 ml of the 1% MA solution was added, stirred and then 20 ml of 0.1 M NaCl 0.05 M sodium phosphate buffer was added. This suspension could be stored at 4 C for a period of up to 4 weeks.

3. Preparation of the methylated-albumin Kieslguhr columns. The column consisted of four layers packed in a 2 cm x 40 cm glass tube equipped with a sintered glass disc. The bottom layer consisted of 1 g of standard grade Whatman paper powder packed by using a suspension in 20 ml of 0.1 M NaCl-0.05 M sodium phosphate buffer, pH 6.7. The second layer was prepared by adding to the column a suspension of 8 g of Kieslguhr in 40 ml of 0.01 M NaCl-0.05 M sodium phosphate, pH 6.7, and 2 ml of a 1% MA solution that was added to the mixture after it was boiled and cooled in an ice bath. The third layer was

prepared by suspending 6 g Kieslguhr in 40 ml of NaCl-0.05 M sodium phosphate buffer, pH 6.7. The suspension was boiled, cooled and combined with 10 ml of MAK just before the mixture was added to the column. The top layer was prepared by suspending 1 g Kieslguhr in 20 ml of 0.1 M NaCl-0.05 M sodium phosphate buffer, pH 6.7, and adding it to the column. All layers were packed using an air pressure of approximately 5 psi. Care was taken to prevent the fluid level from falling below the surface of the column packed by adding each layer when the fluid level was about 0.5 cm above the top of the previous layer. The column was then washed with 150 ml of 0.35 M NaCl-0.05 M sodium phosphate, pH 6.7, the starting buffer of the gradiant system.

4. Chromatography of extracted nucleic acids. The absorbancy of an aliquot from the dialyzed nucleic acid extract was measured at 260 mμ on a Beckman DU spectrophotometer. The number of absorbancy units was calculated as follows:

Number of Absorbancy Units = Extract volume x dilution x absorbancy at 260 mμ.

To keep from overloading a column, approximately 50 absorbancy units of an extract were added to the MAK column. The extract was added to the column in 40 ml of the starting buffer followed by another 15 ml. Nucleic acids were then eluted with a linear-gradient system of an increasing concentration of buffered-NaCl. The gradient was between 0.35 M NaCl-0.05 M sodium phosphate, pH 6.7 and 1.4 M NaCl-0.5 M sodium phosphate, pH 6.7. A fraction collector equipped with a Gilson Medical Electronic 260 mμ scanner and a Texas Instrument Recti-Riter recorder was used to collect 5 ml fractions and to monitor the effluent. The absorbancy of each fraction was measured at 260 mμ with a Beckman DB

spectrophotomer. In cases where P^{32} was used, 2 ml of each fraction were pipetted into a planchet, dried at 60 C and radioactivity was determined using a Nuclear Chicago gas flow counter, model Number D 47, equipped with a thin window for CM counting.

D. Incorporation studies with P^{32}.

Detached calamondin leaves were placed in a $H_3P^{32}O_4$ solution containing 100 uc/ml for 24 hours. With fruits, however, stem uptake was not used because of fruit abscission. In this case the fruits were successfully administered the isotope by placing a 0.1 ml drop of $H_3P^{32}O_4$ (100 uc/ml) on the fruit button, allowing it to be absorbed. This was repeated and followed by a 0.2 ml drop of distilled water to quantitatively wash in the isotope. After leaving the fruit intact for 24 hours after the treatment, the rind was sampled and analyzed for nucleic acids.

RESULTS

1. Relative concentration of various types of nucleic acids in the rind of calamondin fruits at various stages of growth and development.

Marked differences were found between nucleic acids in the rind tissue of calamondin fruits at various stages of development, as may be seen in the elution chromatograms presented in Figs. 4, 5, 6, 7 and 8. Differences in the relative concentration of each nucleic acid fraction and the ratio between s-RNA and r-RNA are shown in Table 1.

Five nucleic acid fractions were found in a typical chromatogram (Fig. 4). In general, fractionation using the MAK column was good enough in resolution to warrant using it to follow changes in these fractions after various treatments. Soluble RNA consisted of three distinct peaks, the first (s_1-RNA) was considerably larger than the other two (s_{2+3}-RNA). However, in Fig. 4 the first peak is almost negligible, as the tissue was so young. The fourth major fraction was that of deoxyribonucleic acid-ribonucleic acid complex (DNA-RNA). The fifth fraction was r-RNA This latter fraction contained a mixture of light and heavy ribosomal material plus possibly m-RNA.

The smallest fruits that were investigated (stage I) were unique in having the DNA-RNA complex and also in showing a very small amount of s_1-RNA (Fig. 4). Rind tissue of older fruits showed a gradual increase in s-RNA and a decrease in the r-RNA (Figs. 5, 6, 7 and 8). These trends are shown graphically in Fig. 9. The ratio between s-RNA and r-RNA is nearly unity by the fourth stage of development,

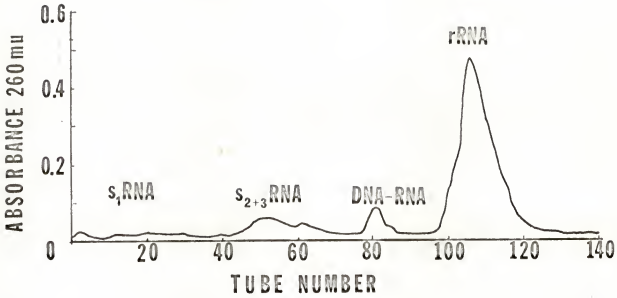


Fig. 4. Chromatographic elution profile of various types of RNA extracted from the rind of green immature calamondin fruits (0.5-1.0 cm diameter) as fractionated on a MAK column.

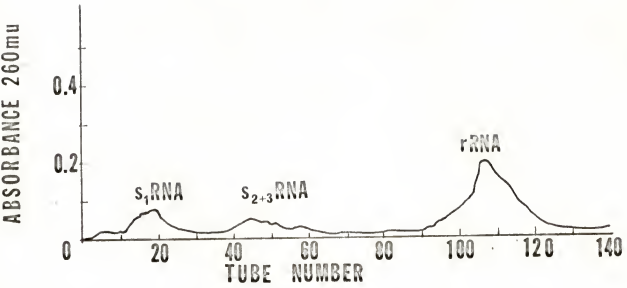


Fig. 5. Chromatographic elution profile of various types of RNA extracted from the rind of green immature calamondin fruits (1.0-1.5 cm diameter) as fractionated on a MAK column.

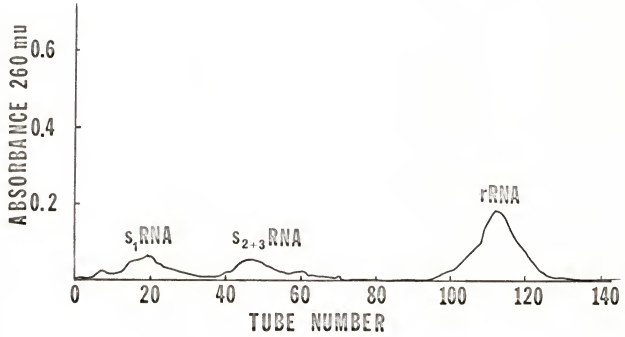


Fig. 6. Chromatographic elution profile of various types of RNA extracted from the rind of green immature calamondin fruits (1.5-2.0 cm diameter) as fractionated on a MAK column.

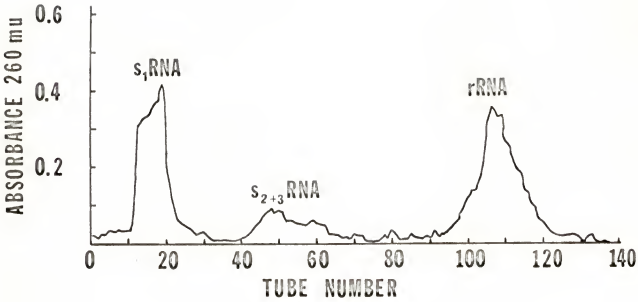


Fig. 7. Chromatographic elution profile of various types of RNA extracted from the rind of green mature calamondin fruits (2.0-2.5 cm diameter) as fractionated on a MAK column.

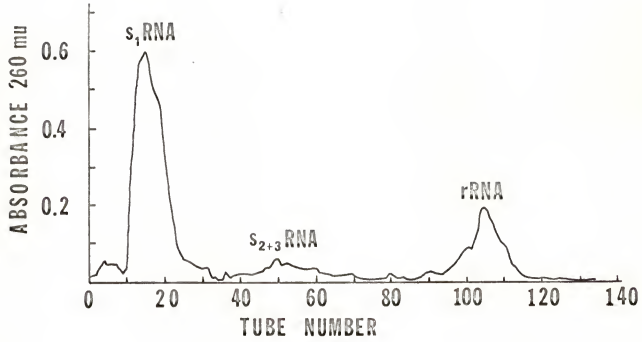


Fig. 8. Chromatographic elution profile of various types of RNA extracted from the rind of fully ripened calamondin fruits (2.0-3.0 cm diameter) as fractionated on a MAK column.

Table 1.—Relative concentration of various nucleic acid fractions extracted from the rind of calamondin fruits at various stages of growth and development.

Fruit diameter (cm)	Absorbancy units /g fresh weight	% of Total absorbancy				
		s_1-RNA	s-RNA**	DNA-RNA	r-RNA	s-RNA / r-RNA Ratio
0.5-1.0	24.22	3.52	17.29	6.77	75.93	0.23
1.0-1.5	22.51	15.50	27.26	0.0	72.74	0.38
1.5-2.0	7.20	17.54	32.90	0.0	67.10	0.50
2.0-2.5	6.76	36.34	49.61	0.0	50.39	0.99
3.0*	3.45	71.45	78.20	0.0	21.83	3.58

*Fruits were fully ripened.
**Sum of s_1-RNA and s_{2+3}-RNA.

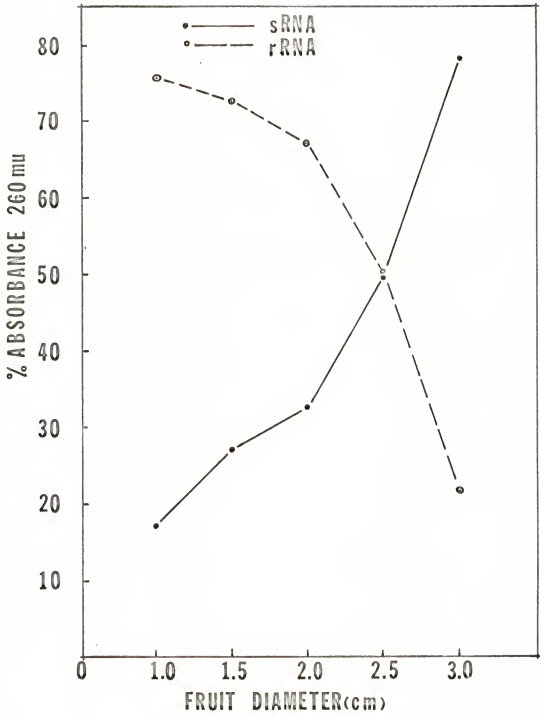


Fig. 9. Changes in the relative concentration of s-RNA and r-RNA in the rind of calamondin fruits at various stages of growth and development.

while it has increased by more than three-fold in ripe fruits due to the sharp increase (57.6%) in s-RNA and the sharp decline (56.7%) in r-RNA. An interconversion may take place between these fractions. Also, total nucleic acids per gram fresh weight decreased sharply with age (Table 1).

2. Effect of the stage of growth and development of the calamondin leaf on the relative concentration of various types of nucleic acids.

A pattern of increasing s-RNA and decreasing r-RNA with age similar to that described for calamondin fruits was found with calamondin leaves. The relative concentration of various types of nucleic acids extracted from young, fully-expanded and senescing calamondin leaves is shown in Table 2 and the elution chromatograms of nucleic acids extracted from the same leaves are shown in Fig. 10. It can be seen that differences in the relative concentrations of s-RNA and r-RNA in mature and senescent leaves were not as substantial as they were in the fruits. The DNA-RNA complex also existed at all three stages of leaf growth and development. Total nucleic acids extracted declined sharply as the leaf aged.

3. Effect of ethylene on the relative concentration of chlorophyll and various types of nucleic acids in calamondin fruit rind.

Ethylene treatment at commercial rates for 24 hours resulted in initiating the abscission precesses of calamondin fruits and leaves. Data in Table 3 demonstrate the progressive influence of ethylene on the chlorophyll content of the fruit rind. The difference in color began to be very pronounced 9 days after the ethylene treatment. But treated and non-treated fruits had very little chlorophyll remaining

Table 2.—Relative concentration of various nucleic acid fractions extracted from calamondin leaves at various stages of growth and development.

Stage of leaf development	Absorbancy units /g fresh weight	% of Total absorbancy				s-RNA r-RNA Ratio
		s_1-RNA	s-RNA*	DNA-RNA	r-RNA	
Young expanding	70.09	2.72	17.95	10.82	71.24	0.26
Fully expanded	22.53	25.42	42.54	7.57	50.88	0.88
Senescing	15.82	34.29	48.04	6.63	45.33	1.11

*Sum of s_1-RNA and s_{2+3} -RNA

Fig. 10. Chromatographic elution profiles of various types of RNA extracted from calamondin leaves at various stages of growth and development.as fractioned on MAK columns.

A: Young expanding.
B: Fully expanded.
C: Senescing.

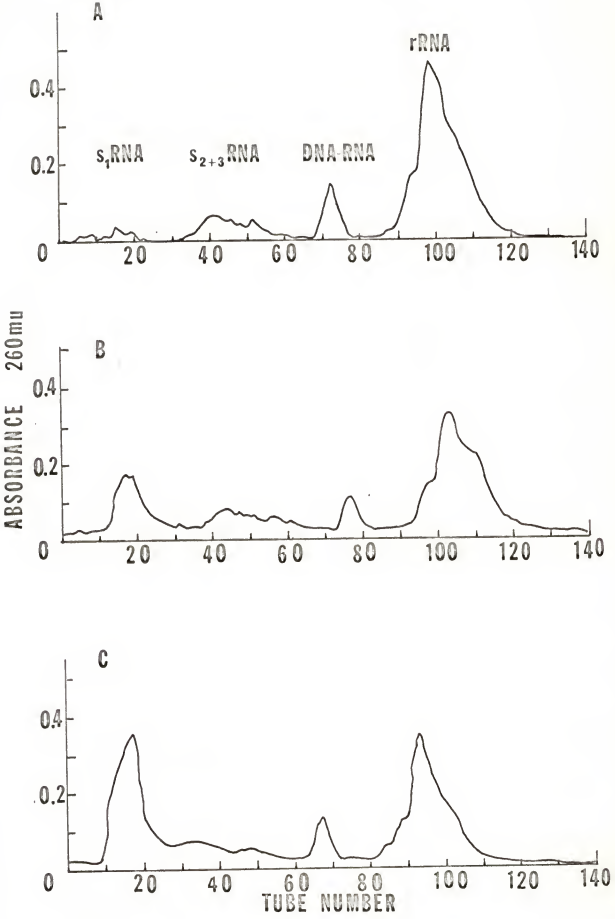

A
rRNA
0.4
0.2
s_1RNA
s_{2+3}RNA
DNA-RNA
0
20
40
60
80
100
120
140
ABSORBANCE 260mu
B
0.4
0.2
0
20
40
60
80
100
120
140
C
0.4
0.2
0
20
40
60
80
100
120
140
TUBE NUMBER

Table 3.—Chlorophyll content in the rind of mature calamondin fruits at various times after treatment with ethylene.

Days after treatment	Treatment	Chlorophyll content ($\mu g/cm^2$)
1	Control	13.63
	Ethylene	11.92
9	Control	7.61
	Ethylene	2.42
18	Control	1.65
	Ethylene	0.89

18 days after treatment.

The relative concentration of various types of nucleic acids of ethylene-treated and non-treated fruits after 1, 9, and 18 days after treatment is shown in Table 4. Ethylene caused a slight decline in the amount of nucleic acids extracted from the rind 1 day after treatment. It also brought about a slight increase in s-RNA and a slight decrease in r-RNA. This was evidenced by both absorbancy measurements at 260 mμ and P^{32} incorporation data (Tables 4 and 5 and Figs. 11 and 12).

Differences in various types of RNA were very striking 9 days after the ethylene treatment as shown in Fig. 13. Ethylene-treated fruits showed only s_1-RNA and very little s_{2+3}-RNA. No DNA-RNA or r-RNA were detected in the extract. Control fruits, however, showed s_1-RNA, s_{2+3}-RNA and r-RNA but no DNA-RNA. At 18 days after the ethylene treatment both treated and non-treated fruits exhibited only s_1-RNA. Fig. 14 represents the elution chromatograms of both treated and non-treated fruits since both were almost identical. Chlorophyll level in both treated and non-treated fruits dropped to a very low level and the fruits lost almost all their green color 18 days after treatment.

4. Effect of ethylene on the relative concentration of various types of nucleic acids, in fully expanded, mature leaves of calamondin.

Ethylene did not seem to have any immediate effect on the relative concentration of various types of RNA extracted from mature leaves of calamondin. The relative concentrations of various types of RNA extracted from calamondin leaves, one day after treatment with ethylene

Table 4.—Relative concentration of various nucleic acid fractions extracted from the rind of mature calamondin fruits at various times after treatment with ethylene.

Treatment	Days after treatment	Absorbancy units /g fresh weight	% of Total absorbancy				s-RNA / r-RNA Ratio
			s_1-RNA	s-RNA*	DNA-RNA	r-RNA	
Control	1	12.10	48.54	55.36	0.0	44.64	1.24
Ethylene		8.34	54.57	63.33	0.0	36.67	1.73
Control	9	10.30	44.93	47.76	0.0	42.24	1.37
Ethylene		7.13	91.90	100.00	0.0	0.0	—
Control	18	12.10	100.00	100.00	0.0	0.0	—
Ethylene		11.60	100.00	100.00	0.0	0.0	—

*Sum of s_1-RNA and s_{2+3}-RNA.

Table 5.—P^{32} incorporation into various nucleic acid fractions extracted from the rind of mature calamondin fruits at various times after treatment with ethylene.

Treatment	Days after treatment	% of Total radioacticity (CPM) incorporated into various nucleic acid fractions		
		s-RNA*	DNA-RNA	r-RNA
Control	1	60.09	0.0	39.91
Ethylene		64.17	0.0	35.83
Control	9	82.58	0.0	17.42
Ethylene		100.00	0.0	0.0
Control	18	100.00	0.0	0.0
Ethylene		100.00	0.0	0.0

*Sum of s_1-RNA and s_{2+3}-RNA.

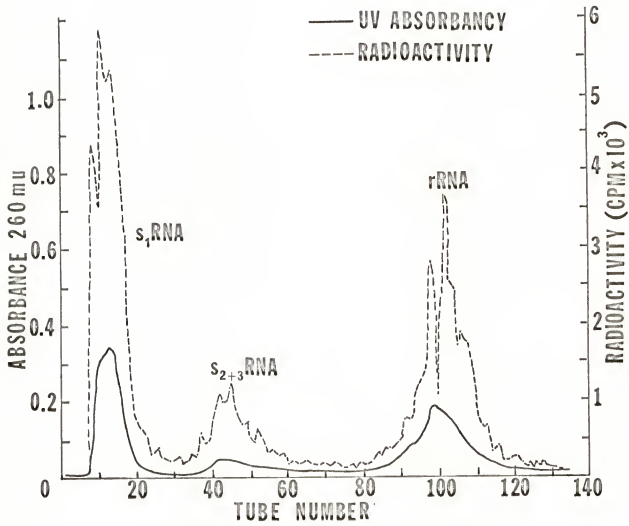


Fig. 11. Chromatographic elution profile of various types of P^{32}-labeled RNA extracted from the rind of mature green calamondin fruits 1 day after excision. Fractionation was made on a MAK column. Absorbancy and radioactivity were determined on the same sample.

Fig. 13. Chromatographic elution profiles of various types of P^{32}-labeled RNA extracted from the rind of control (A) and ethylene-treated (B) mature green calamondin fruits 9 days after treatment. Fractionation was made on MAK columns. Absorbancy and radioactivity were determined on the same sample.

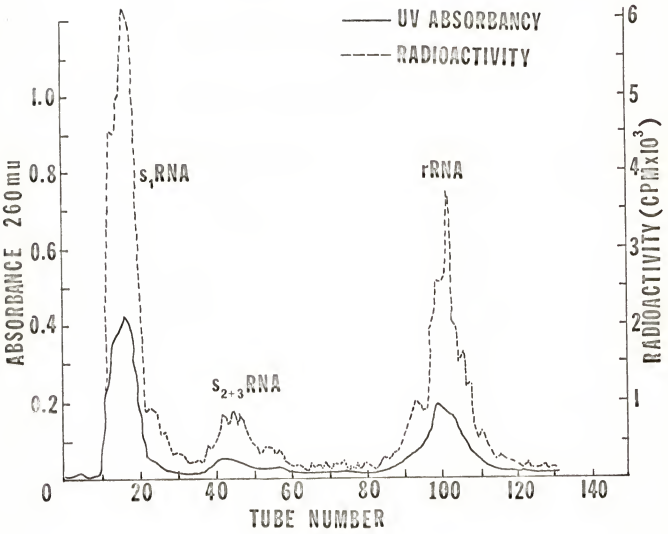
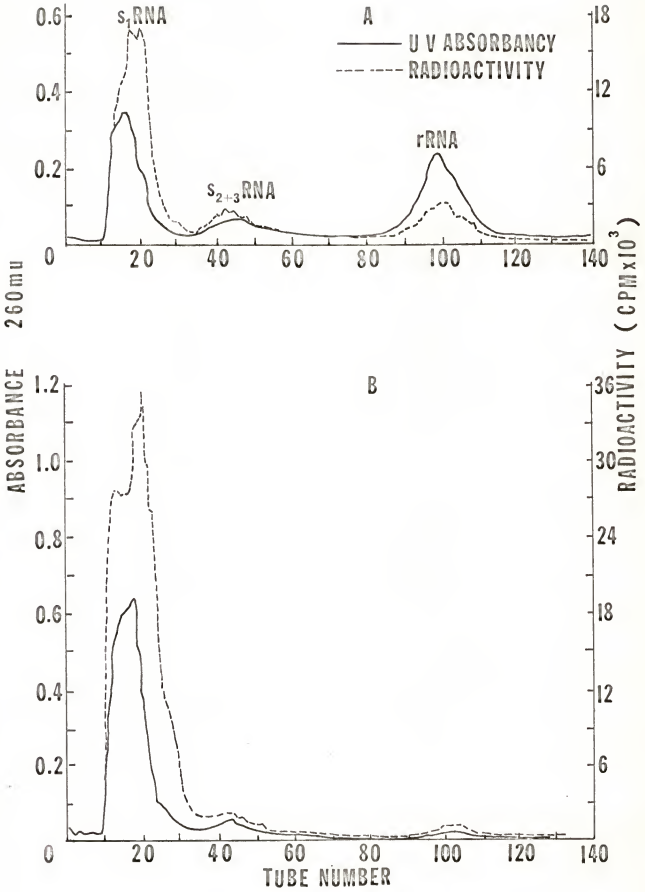


Fig. 12. Chromatographic elution profile of various types of P^{32}-labeled RNA extracted from the rind of mature green calamondin fruits 1 day after treatment with ethylene. Fractionation was made on a MAK column. Absorbancy and radioactivity were determined on the same sample.


A
s_1RNA
s_{2+3}RNA
rRNA
U V ABSORBANCY
RADIOACTIVITY
260mu
RADIOACTIVITY (CPMx10^3)
B
ABSORBANCE
TUBE NUMBER

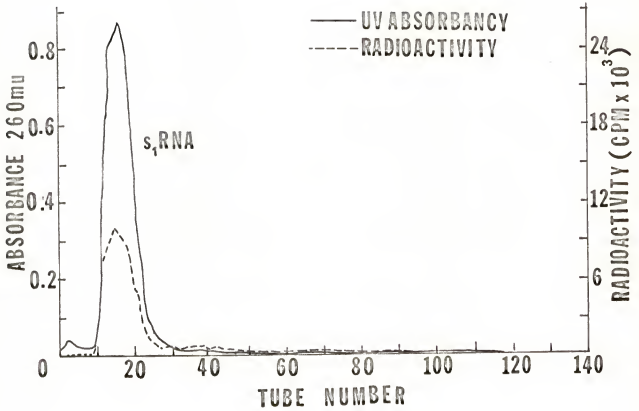


Fig. 14. Chromatographic elution profile of various types of P^{32}-labeled RNA extracted from the rind of mature green calamondin fruits 18 days after treatment with ethylene. Fractionation was made on a MAK column. Absorbancy and radioactivity were determined on the same sample.

are given in Table 6. Absorbancy measurements at 260 mμ showed that the s-RNA, the DNA-RNA complex and the r-RNA fractions remained almost unchanged, however, as shown by the first column in the table. There was a decline of about 22% in the yield of total extractable RNA after the 1-day ethylene treatment.

Although ethylene did not influence the relative concentrations of various types of nucleic acid, it apparently caused an increase in the rate of turnover of certain types (Fig. 15). Ethylene had a striking influence on the incorporation of P^{32} into the DNA-RNA complex of the leaves after 1-day exposure (Fig. 15 and Table 7). These data indicate that ethylene has a promotive action on the rate of turnover of the DNA-RNA and s-RNA fractions.

5. Effect of GA and light on color changes in the rind of three sweet orange cultivars.

Preliminary experiments to establish whether GA could or could not delay senescence of detached citrus fruits, as it does with fruits attached to the tree (29, 30, 31), were conducted with 'Parson Brown', 'Pineapple' and 'Valencia' fruits. Interactions between GA and light were also checked since light plays a role in chlorophyll synthesis (101) and chlorophyll was the component measured.

'Parson Brown' fruits treated with GA showed significantly higher levels of chlorophyll than the untreated control at all three dates of analysis (Fig. 16 and Table 8) even under 2 ft-c light intensity. However, the apparent increase in chlorophyll content 10 days after the GA treatment was not significant. Apparently no interaction took place between GA and light (Table 8). Similar findings were reported by Lewis

Table 6.—Relative concentration of various nucleic acid fractions extracted from fully expanded mature leaves of calamondin after treatment with ethylene[1].

Treatment	Absorbancy units /g fresh weight	% of Total absorbancy				s-RNA r-RNA Ratio
		s_1-RNA	s-RNA[2]	DNA-RNA	r-RNA	
Control	34.83	35.40	47.61	5.85	46.54	0.76
Ethylene	27.00	33.86	47.94	5.13	46.92	0.72

[1]Ethylene treatment was for 1 day and the leaves sampled immediately after the treatment.

[2]Sum of s_1-RNA and s_{2+3}-RNA.

Fig. 15. Chromatographic elution profiles of various types of P^{32}-labeled RNA extracted from control (A) and ethylene-treated (B) fully-expanded healthy calamondin leaves 1 day after treatment. Fractionation was made on MAK columns. Absorbancy and radioactivity were determined on the same sample.

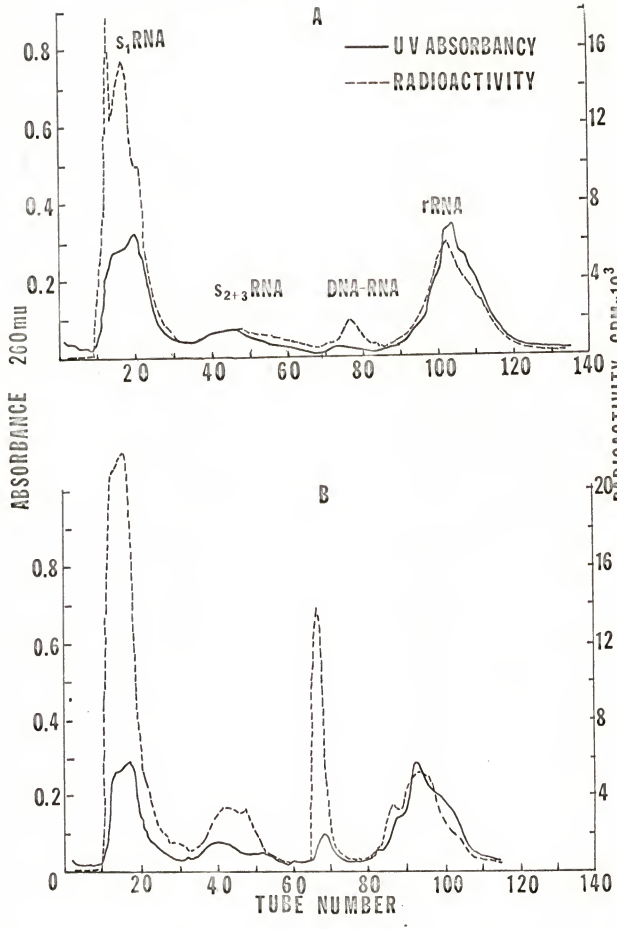

A
B
UV ABSORBANCY
RADIOACTIVITY
s_1RNA
s_{2+3}RNA
DNA-RNA
rRNA
ABSORBANCE 260mμ
RADIOACTIVITY CPMx10^3
TUBE NUMBER
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
140
16
12
8
4

Table 7.—P^{32} incorporation into various nucleic acid fractions extracted from fully expanded mature leaves of calamondin after treatment with ethylene[1].

Treatment	% of total radioactivity (CPM) incorporated into various nucleic acid fractions		
	s-RNA[2]	DNA-RNA	r-RNA
Control	70.33	0.9	28.73
Ethylene	67.15	10.85	22.00

[1]Ethylene treatment was for 1 day and the leaves sampled immediately after the treatment.

[2]Sum of s_1-RNA and s_{2+3}-RNA.

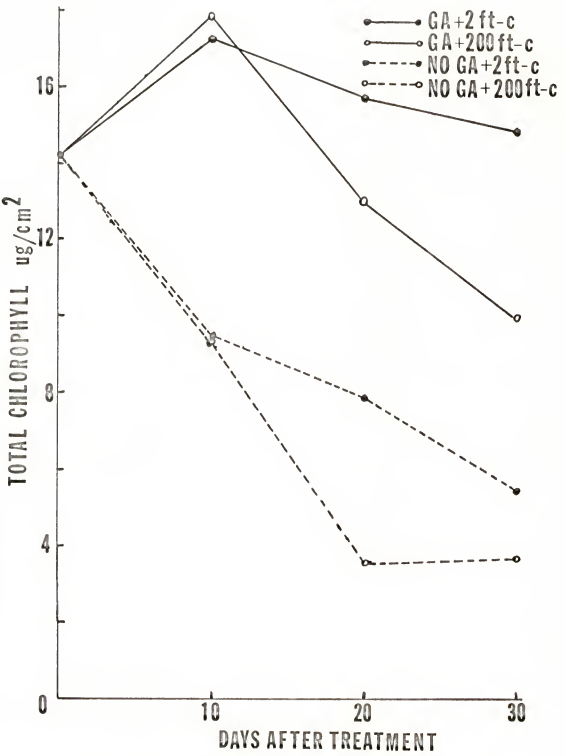


Fig. 16. Effect of GA and light intensity on chlorophyll content in the rind of mature green 'Parson Brown' orange fruits.

Table 8.—Analysis of variance for data on the effect of gibberellic acid, light, intensity and time on total chlorophyll content of the rind of mature green 'Parson Brown' fruits.

Source of variation	df	Mean square	F[1]
Total	15	21.44	
Days	3	30.66	4.17 ***
GA	3	54.50	7.41 ***
A vs. B	1	11.01	1.50
GA vs. No GA	1	152.46	20.74 ***
L vs. GA	1	0.03	0.004
Error	9	7.35	

[1] *** = Significant at 1% level

A = 200 ft-c.

B = 2 ft-c.

Coggins (85) on 'Washington' navel orange.

GA-treated 'Valencia' fruits maintained higher levels of chlorophyll in the dark, a treatment that should prevent chlorophyll synthesis (101), than the untreated fruits. Reflectance values at 675 mμ of GA-treated fruits decreased from 0.75 to 0.50 over a period of 5 weeks, whereas, the readings of non-treated 'Valencia' fruits decreased from 0.77 to 0.24 during the same period of time as shown in Table 9. This indicates that GA prevents chlorophyll destruction in the rind of 'Valencia' fruits. Analysis of variance (Table 10) illustrates that time and GA were the most significant factors contributing to the differences in rind color. GA concentrations ranging from 10 to 1000 ppm had approximately the same effect on the degree of loss of green color from the rind of 'Valencia' oranges (Table 11). The reflectance values at 675 mμ measured over a period of 5 weeks were nearly the same for all treated fruits. However, all concentrations of GA utilized resulted in increased chlorophyll content over the controls after 5 weeks of storage (Table 12).

Fully ripened 'Parson Brown' and 'Pineapple' fruits treated with GA showed no detectable change in color while non-treated fruits continued to lose chlorophyll measured as reflectance at 675 mμ (Table 13). GA seems to have no significant effect on color change in the rind of 'Parson Brown' fruits (Table 14). This could be due to the relatively large variation in the content of chlorophyll between fruit samples that were selected randomly at the beginning of the experiment. GA did, however, have a significant effect on color differences between treated and non-treated 'Pineapple' fruits (Table 15).

Table 9.—Reflectance values of the rind of mature green 'Valencia' orange fruits as influenced by gibberellic acid, light and time.

Weeks after treatment	Average reflectance[1] X 100 at 675 mμ			
	GA + Dark	GA + Light	No GA + Dark	No GA + Light
0	75	75	77	74
1	57	58	56	38
2	56	57	42	31
3	46	58	39	31
4	46	55	43	31
5	50	49	24	31

[1] Reflectance values were obtained with a Bausch & Lomb "Spectronic 20" spectrophotometer equipped with a reflectance unit.

Table 10.—Analysis of variance for data on the effect of gibberellic acid, light and time on reflectance values of the rind of 'Valencia' orange.

Source of variation	df	Mean square	F[1]
Total	23	231.35	
Weeks	5	681.73	17.23 ***
Treatment	3	439.57	11.11 ***
Dark vs. Light	1	25.42	0.64
GA vs. No. GA	1	1105.68	27.94 ***
Interaction	1	178.60	4.74 **
Error	1	34.57	

[1] *** = Significant at 1% level

** = Significant at 5% level

Table 11.—Reflectance values of the rind of mature green 'Valencia' orange fruits at various times after treatment with various concentrations of gibberellic acid.

Weeks after treatment	Average reflectance[1] X 100 at 675 mμ						
	GA concentration (ppm)						
	0	10	30	60	100	500	1000
0	77	77	78	79	78	75	78
1	47	61	58	68	60	60	60
2	39	57	53	58	56	58	57
3	38	55	51	56	54	58	56
4	33	51	47	51	51	57	54
5	32	51	47	50	49	53	51

[1] Reflectance values were obtained with a Bausch & Lomb "Spectronic 20" spectrophotometer equipped with a reflectance unit.

Table 12.—Analysis of variance for data on the effect of various concentrations of gibberellic acid on reflectance values of the rind of mature green 'Valencia' orange.

Source of variation	df	Mean square	F[1]
Total	125	138.02	
Weeks	5	2489.02	89.31 ***
Concentration	6	556.31	19.96 ***
W x Conc.	30	27.87	0.85
Sample/conc.	14	32.67	2.96 ***
W x Sample/conc.	70	2.52	

[1]*** = Significant at 1% level

Table 13.—Color change in the rind of fully ripened 'Parson Brown' and 'Pineapple' orange fruits at various times after gibberellic acid treatment.

Weeks after treatment	Reflectance[1] X 100 at 675 mμ			
	'Parson Brown'		'Pineapple'	
	GA-treated	Control	GA-treated	Control
0	26.5	32.4	16.1	15.9
1	23.5	27.7	14.8	13.2
2	23.1	24.3	15.0	13.0
3	25.1	26.1	17.6	14.1
4	24.0	24.8	16.4	13.4
5	26.3	23.1	17.3	14.5

[1]Reflectance values were obtained with a Bausch & Lomb "Spectronic 20" spectrophotometer equipped with a reflectance unit.

Table 14.—Analysis of variance for data on the effect of gibberellic acid and time on color change in the rind of 'Parson Brown' orange fruits.

Source of variation	df	Mean square	F
Total	35	9.49	
Week	5	26.74	1.69
Treatment	1	25.33	1.60
W x GA	5	15.79	4.84
Sample/treat.	4	3.26	0.80
W x sample/treat.	20	4.05	

Table 15.—Analysis of variance for data on the effect of gibberellic acid and time on color change in the rind of 'Pineapple' orange fruits.

Source of variation	df	Mean square	F
Total	35	9.04	
Week	5	5.36	2.58
Treatment	1	42.68	20.52 ***
W x GA	5	2.08	0.05
Sample/treat.	4	41.64	11.90 ***
W x Sample/treat.	20	3.50	

*** = Significant at 1% level

6. Effect of gibberellic acid on chlorophyll and polymeric nucleic acids of calamondin fruits

After it was observed that GA delayed degreening of detached fruits of all three sweet orange cultivars, analyses were made of chlorophyll and nucleic acids in calamondin fruits. It was found that GA delayed the destruction of chlorophyll in these fruits in a pattern similar to that of the three sweet orange cultivars tested (Fig. 17 and Table 16).

Polymeric nucleic acid changes in these same fruits are shown in Figs. 18, 19, 20 and 21 and in Tables 17 and 18. When the chlorophyll of the controls started to decline, 14 days after the treatment (Table 16) they were analyzed for nucleic acids and were found to comtain only s_1-RNA. Comparable GA-treated fruits contained polymeric groups of s_1-RNA, s_{2+3}-RNA and r-RNA. The DNA-RNA complex was not detected in either sample at 14 days after treatment. After 28 days, GA-treated fruits were still green while untreated fruits had lost most of their chlorophyll (Table 16 and Fig. 17). However, analyses of both treated fruits and controls for nucleic acids demonstrated that they contained primarily s_1-RNA (Figs. 21 and Table 17).

The P^{32} incorporation experiments substantiated the findings with extracts of non-labeled polymeric nucleotides (compare Figs. 18, 19, 20 and 21 and Table 18). This demonstrates that the pattern of P^{32} incorporation into the fractions followed the pattern detected by absorbance measurements (Table 17).

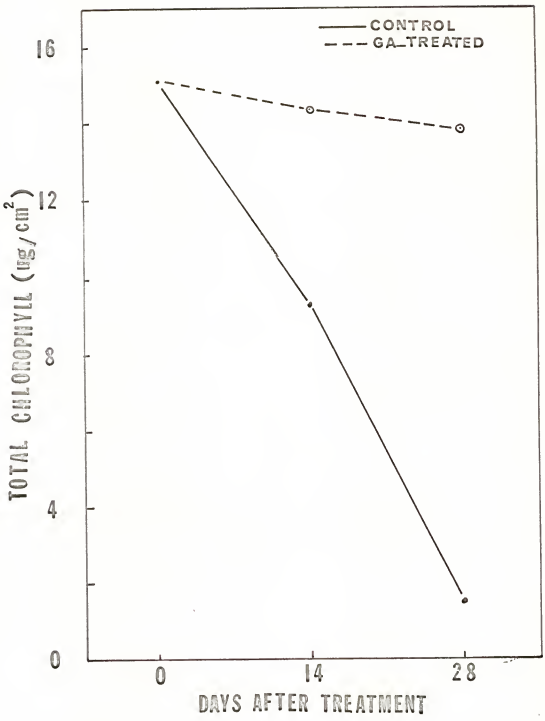


Fig. 17. Effect of gibberellic acid and time on chlorophyll concentration in the rind of mature green calamondin fruits.

Table 16.—Chlorophyll contration in the rind of mature calamondin fruits at various times after treatment with gibberellic acid.

Days after treatment	Treatment	Chlorophyll content ($\mu g/cm^2$)
0	Control	15.14
14	Control	9.30
	GA	14.35
28	Control	1.51
	GA	13.80

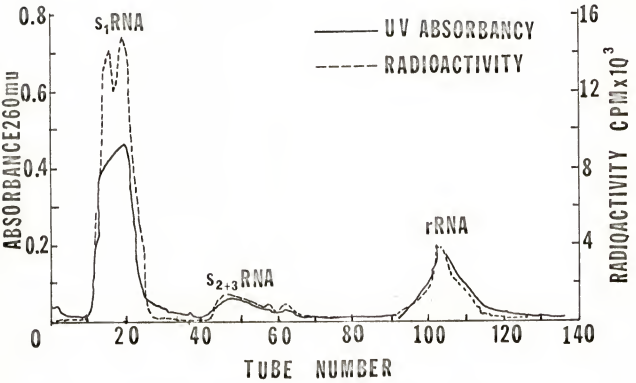


Fig. 18. Chromatographic elution profile of various types P^{32}-labeled RNA extracted from the rind of mature green calamondin fruits 1 day after excision. Fractionation was made on a MAK column. Absorbancy and radioactivity were determined on the same sample.

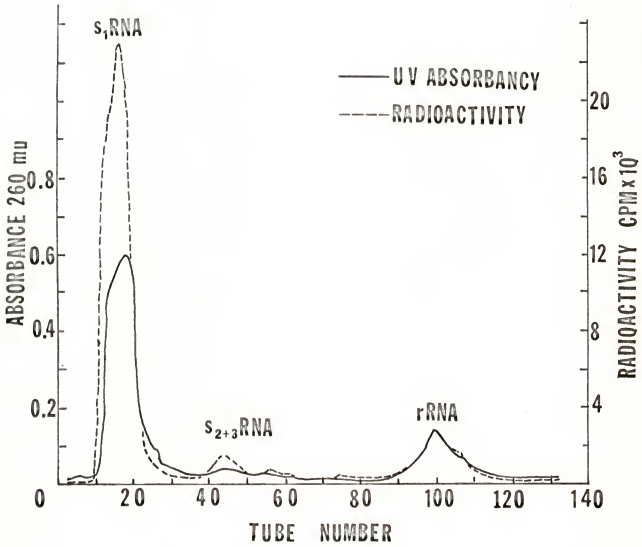


Fig. 19. Chromatographic elution profile of various types of P^{32}-labeled RNA extracted from the rind of GA-treated mature green calamondin fruits 14 days after treatment. Fractionation was made on a MAK column. Absorbancy and radioactivity were determined on the same sample.

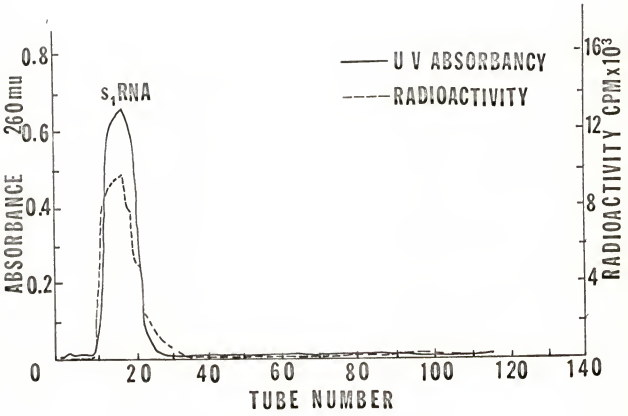


Fig. 20. Chromatographic elution profile of various types of P^{32}-labeled RNA extracted from the rind of mature green calamondin fruits 14 days after excision. Fractionation was made on a MAK column. Absorbancy and radioactivity were determined on the same sample.

Fig. 21. Chromatographic elution profiles of various types of P^{32}-labeled RNA extracted from the rind of GA-treated (A) and control (B) mature green calamondin fruits 28 days after treatment. Fractionation was made on MAK columns. Absorbancy and radioactivity were determined on the same sample.

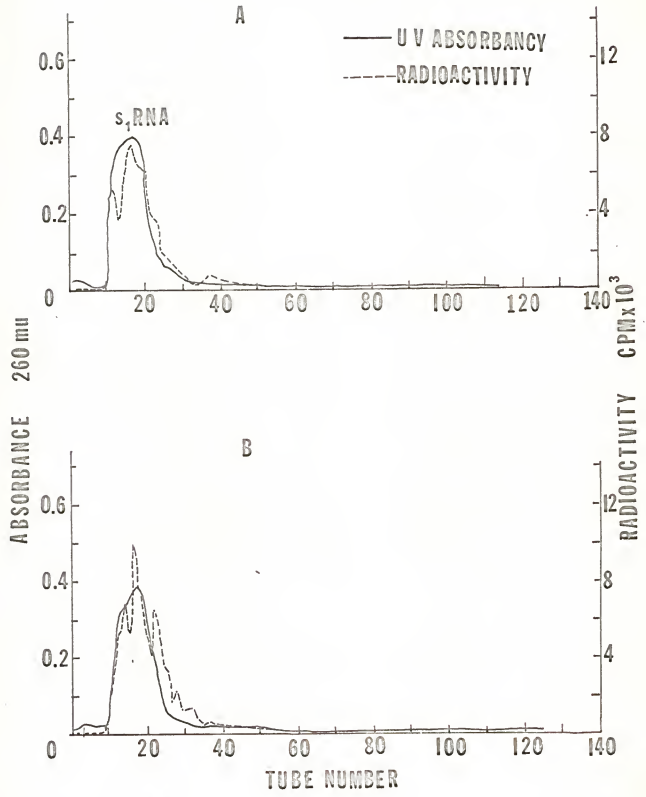

A
U V ABSORBANCY
RADIOACTIVITY
s_1RNA
0.6
0.4
0.2
0
20
40
60
80
100
120
140
12
8
4
$CPMx10^3$
ABSORBANCE 260 mμ
B
RADIOACTIVITY
TUBE NUMBER

Table 17.—Relative concentration of various nucleic acid fractions in the rind of mature calamondin fruits at various times after treatment with gibberellic acid.

Treatment	Days after treatment	Absorbancy units /g fresh weight	% of Total absorbance			s-RNA / r-RNA Ratio
			s_1-RNA	s-RNA*	r-RNA	
Control	0	13.00	63.50	71.38	28.62	2.49
Control	14	12.83	100.0	100.0	0.0	0.0
GA		17.00	73.59	77.69	22.31	3.48
Control	28	4.07	100.00	0.0	0.0	—
GA		5.30	100.00	0.0	0.0	—

*Sum of s_1-RNA and s_{2+3}-RNA.

Table 18.—P^{32} incorporation into various nucleic acid fractions of the rind of mature green calamondin fruits at various times after treatment with gibberellic acid.

Treatment	Days after treatment	% of Total radioactivity (CPM) incorporated into various nucleic acid fractions		
		s-RNA*	DNA-RNA	r-RNA
Control	0	79.47	0.0	20.53
Control	14	100.00	0.0	0.0
GA	14	87.67	0.0	12.33
Control	28	100.00	0.0	0.0
GA	28	100.00	0.0	0.0

*Sum of s_1-RNA and s_{2+3}-RNA.

DISCUSSION

The method of extracting nucleic acids developed by Kirby in 1959 (77), and utilized by many workers (12, 21, 68, 168) proved to be unsuitable for citrus rind tissue. Many extraction trials using this method were made on rind tissue from 'Hamlin' and 'Valencia' orange and from calamondin fruits. Nucleic acid extracts contained mianly s-RNA, with very little of DNA-RNA and r-RNA. The method was then modified by grinding the tough rind tissue in an Omni-mixer and then regrinding the residue and interphase layer with a mortar and pestle for 15 minutes.

Any method of grinding for the extraction of nucleic acids should very thoroughly rupture the cells and the particulate components of the cytoplasm in order to expose the nucleic acids to the extractants. In studies on nucleic acids in relatively tender plant materials such as soybean (73) and pea seedlings (12) and peanut cotyledons (21, 23) the use of a tissue grinder or an Omni-mixer proved to be quite adequate for grinding of tissues. In other studies (42, 103) on DNA and RNA extraction from leaves of decidious woody plants, the tissue was freeze-dried and then ground in a Wiley mill. But due to the leathery texture of many citrus fruit rinds (117, 178) the grinding procedure for this study had to be modified with the use of a mortar and pestle in a second grinding for at least 15 minutes in order to crush larger particles left after the first grinding. This yielded extracts with components that resembled those found in succulent tissues.

Amount of reagents used were also increased to improve the extraction of nucleic acids from the subcellular structures. Ten ml of sodium lauryl sulfate was used instead of 3 ml as recommended by Cherry (21) resulted in the extraction of r-RNA. The extraction was also improved by increasing the amount of the bentonite suspension used. Bentonite has been shown to inhibit the action of ribonuclease by surface adsorption (13).

Even with the modified extraction procedure, as outlined above, there was the contamination of the final nucleic acid extract. These contaminants along with some traces of phenol resulted in a shift in the absorbancy maximum from the usual 260 mμ to 270 mμ. To rid the final nucleic acid extract from these contaminants, the extraction procedure was further modified by precipitating in absolute ethanol twice rather than only once (12, 21). Bendana et al. (6) found it necessary to do the precipitation four times to obtain a pure nucleic acid extract free of all contaminants from pea stem sections.

The extraction method adopted in this work was successful in releasing s_1-RNA, s_{2+3}-RNA, DNA-RNA from calamondin leaves at the three stages of development. However, applying the same procedure to the rind of calamondin fruits, it was possible to extract only the three types of RNA and no DNA-RNA was extracted except from the rind of very young fruits (stage I). Here, it was in small quantities. Failure to extract the DNA-RNA from more mature fruits could be attributed partially to the fact that it existed at very low concentration in the very young fruits and decreased with age. If this was the case, DNA-RNA in citrus fruits may have a short half life as in peanut

cotyledons (21). The third possibility is that there could have been some degradation by nucleases during grinding at a time when RNA was not yet protected by phenol and bentonite as suggested by Mach and Vassalli (92). However, this seems unlikely since the initial grinding of the tissue was at 3 C for only 5 minutes. Furthermore, DNA-RNA would have to have been preferentially destroyed and this polymeric component is generally the most resistant to nucleases (94).

The two methods used for chlorophyll assays in this study were shown to be reliable (30, 117, 158). Reflectance measurement at 675 mμ was used on citrus fruits by Oberbacher (116) and a very high degree of correlation was obtained between reflectance values measured with the "Spectronic 20" and the actual chlorophyll content extracted from the same spot on the rind (117). Using this method, it was possible to measure the color of a fruit for several weeks without sacrificing the tissue.

GA was found to retard the disappearance of chlorophyll from the rind of green 'Parson Brown' and 'Valencia' orange and calamondin fruits. In all cases the chlorophyll content of both GA-treated and control fruits declined but the rate of decline was greater in the control than in the treated fruits. Similar findings have been reported for 'Washington' navel orange (26, 29, 85), grapefruit (31), 'Lisbon' lemon (28) and 'Clementine' mandarin (159). The effect of GA on maintaining the green color of citrus fruits is not fully understood, although, it has been previously suggested that it promotes chlorophyll synthesis (30). From this study, it would seem that GA minimizes chlorophyll degradation. This is supported by the tests

on GA-treated 'Valencia' fruits stored in the dark for 5 weeks. In this case, no chlorophyll should be synthesized since this is a light-dependent process (101). Yet chlorophyll was maintained at a higher level in GA-treated fruits than in non-treated fruits.

Since chlorophyll degradation is a readily discernible and possibly a major characteristic of senescence in higher plants, the influence of GA upon the loss of chlorophyll from mature green citrus fruits is considered as evidence that GA retards senescence of certain citrus fruits. This observation tends to corroborate reports that GA reduces the incidence of rind staining, sticky rind and water spot in the rind of 'Washington' navel orange fruits (32, 33, 34). Rind staining becomes more frequent in 'Washington' navel oranges late in the season (34); therefore, reduction of rind staining by GA could be regarded as a retardation of senescence. Similarly, preharvest sprays of GA retarded senescence by delaying ripening and color change of Kaki fruits (78).

Treatment with 10 ppm of GA was as effective in retarding chlorophyll destruction in the rind of 'Valencia' orange fruits as a concentration of 1000 ppm. This finding did not agree with those reported by Coggins and Hield (27) who found that green color of 'Valencia' fruits was proportional to increased GA concentration up to 300 ppm. However, it should be noted that in their experiment the treatments were conducted on fruits attached to the tree, in which case, GA may have been metabolised and/or translocated (31), or this could be a characteristic that results from an interaction between the tree and the fruit after they were treated.

When orange-colored 'Parson Brown' and 'Pineapple' fruits were treated after harvest with GA, no significant regreening was detected during 5 weeks of storage. These results are in agreement with those reported on 'Washington' navel orange (29), and in contrast to those reported for 'Valencia' orange (30) and grapefruit (31). However, here again the fruits were treated on the trees. Yet, this does indicate that various citrus fruits respond differently to GA treatment, and that gibberellin-induced regreening may take place only in those cultivars that have a natural tendency to regreen in the spring such as 'Valencia' orange and certain grapefruit cultivars.

The action of GA on calamondin fruits does not seem to be confined to the maintenance of a high level of chlorophyll in the fruit rind but also causes the fruits to maintain higher levels of polymeric nucleic acids. Significantly, GA-treated fruits contained r-RNA at 14 days after the treatment where none was found in the controls. This maintenance of r-RNA in GA-treated fruits could result in higher levels of chlorophyll since the latter characteristic is associated with high protein levels (84, 126, 151) and protein levels are very dependent on r-RNA (41, 121). This may indicate that the protein content of GA-treated calamondin fruits may have been increased by the treatment. Fletcher and Osborne (45) reported that GA retarded senescence of Taraxacum leaves and this was associated with higher levels in the tissue of chlorophyll, protein, and RNA. Beevers (5) reported similar findings on GA-treated Nasturtium leaves. GA has also been found to stimulate DNA, DNA-RNA and RNA synthesis in the epicotyl tissue of lentil seedlings (114).

Twenty-eight days after the GA treatment, both treated and non-treated fruits contained only s-RNA and no r-RNA was detected despite the fact that GA-treated fruits were still green while those of the control had very little chlorophyll. It seems that the effectiveness of GA in maintaining the integrity of r-RNA has lasted only from 9 to 14 days (Figs. 13, 20 and Tables 4 and 17).

Data on P^{32} incorporation studies (Table 18) demonstrated the close parallelism between absorbance and radioactivity measurements. They showed more P^{32} was incorporated into s-RNA and less into r-RNA as the fruit ripened. Control fruits, analyzed 14 days after treatment showed no radioactivity in the r-RNA fraction, while radioactivity was incorporated in both the s-RNA and r-RNA fractions of GA-treated fruits. In both treated and control fruits, radioactivity was detected 28 days after treatment only in the s-RNA fraction. Data on P^{32} incorporation and those on absorbancy were in close agreement (compare Tables 17 and 18). This may indicate that the system or systems responsible for the synthesis of polymeric nucleic acids of higher molecular weights, e.g., DNA-RNA and r-RNA, may have been impaired as the fruit senesces. It may also indicate that GA may have some effect on maintaining a higher level of r-RNA up to 14 days after fruit excision. The fact that GA-treated fruits were still green in the absence of detectable r-RNA 28 days after treatment may be due to one or both of the following possibilities. It is possible that GA, by maintaining a higher level of chlorophyll in the rind of mature calamondin fruits, may act on some factor or factors that maintain the integrity of the ribosomes. This could result in a higher level of

r-RNA which could in turn maintain a higher level of protein necessary for biosynthesis and/or conservation of chlorophyll. Once the necessary protein has been provided, a higher level of chlorophyll would be maintained despite the absence of r-RNA. The other possibility is that GA may act directly on the chloroplasts to maintain the integrity of its DNA, RNA and protein synthesizing systems, thus resulting in chlorophyll synthesis and/or preservation. The presence of DNA, RNA and protein in the chloroplast has been widely reported (10, 18, 95, 96, 133). Chapman, et al. (18) have reported the presence of DNA, RNA and a system for protein synthesis in the chloroplasts of Acetabularia mediterranea, and others have shown there is a relation between protein and functional chloroplasts (95, 96, 133).

The possibilities that r-RNA could have been degraded during extraction or that it may not have been extracted at all, are ruled out, since the extraction was conducted at low temperature and r-RNA was extracted from both leaves and fruit rind of citrus using the same procedure. It is suggested that r-RNA is naturally degraded during senescence of the rind. The reduction in the number of ribosomes with age has been previously reported (20, 90, 119). Shaw et al. (151) demonstrated electronmicrographically that the ribosomal particles of mesophyll cells of wheat leaves disintegrated and disappeared completely after 4 to 5 days from excision. Sturani and Cocucci (164) also reported that the ribosomes of castor bean endosperm disappeared completely as the endosperm completed its development. Reductions in r-RNA and increases in s-RNA with age have been reported by Cherry

et al. (23) on peanut cotyledons and Ying (190) on pea stems. On the other hand, with healthy systems, Nitsan and Lang (114) found that GA increased the amount of r-RNA in the epicotyl tissue of lentil seedlings. In this case, the majority of the cells in the system were in an active state of development.

The drastic decline in r-RNA and the equally dramatic increase in s-RNA in the leaves and fruit rind of calamondin indicate that a substantial destruction of the ribosomal particles took place with age. Similar findings have been widely reported (20, 23, 90, 119, 122, 151, 152, 161, 164). A change of this nature may be responsible for the drastic decline in protein content associated with senescence of various plant organs (45, 126, 132, 138, 151, 179, 189). The massive destruction of ribosomes has been suggested to result from increased activity of ribonuclease (20, 70, 161). It could also result from impairment of RNA synthesis that provides RNA for the formation of new ribosomes. In either case, protein synthesis would be impaired. Osborne (126) has showed that senescent leaf discs of Xanthium have a reduced capacity to incorporate C^{14}-leucine into protein and C^{14}-orotic acid into RNA. Rogers (140) showed that excised roots, stems and leaves were capable of synthesizing amino acids when fed C^{14}-sucrose or C^{14}-acetate. Racusen (135) demonstrated that the ability of soybean leaves to incorporate amino acids into protein decreases as the time between excision and exposure to $C^{14}O_2$ increases. Breuer and Floriné (11) reported that the activity of ribosomal particles isolated from rat thigh muscle in incorporating labeled amino acids into protein decreased with the age of the animal. These experiments indicate that

the decline in protein content associated with senescence may be due both to increased proteolysis and impaired synthesis. It is suggested that the reduction in r-RNA in calamondin fruits and leaves leads to a decline in the number of ribosomal particles on which amino acids have to be assembled into protein. It is possible that the preferential destruction of certain polyribosomes necessary for the synthesis of certain protein molecules, e.g., an enzyme that is necessary for the synthesis of certain cell components, or the persistence of certain polyribosomes needed for the synthesis of proteolytic enzymes and nucleases, could take place during senescence. Generally, this could result in increased catabolic and decreased anabolic activities.

Senescence of citrus organs was considerably accelerated as indicated by the rapid decline in chlorophyll content and by abscission of all fruits and leaves after treatment with ethylene. Abscission has been regarded as a process allied to senescence (19). s-RNA had increased slightly in the tissue 1 day after exposure to ethylene, but thereafter it has greatly increased. In the meantime, r-RNA had declined slightly by 1 day after a day exposure to ethylene and had completely disappeared by the 9th day. This shift in polymeric nucleotides from higher molecular weight to lower molecular weight fractions reveals the destructiveness of ethylene on cells. A change of this nature could result in complete cessation of protein synthesis. Apparently, destruction of r-RNA and loss of the mechanisms for resynthesis of r-RNA took place sometimes between the 1st and 9th day after the treatment. It seems possible that the action of ethylene may be partially through its influence on nucleic acids and subsequently proteins. Abeles (1) reported that the administration of actinomycin D

to bean, corn and Helianthus seedling tissue inhibited auxin-induced ethylene production. More recently, Abeles and Holmes (2) reported that actinomycin D and cyclohexamide inhibited ethylene-induced abscission of bean explants when they were injected before the ethylene treatment. This indicated that the synthesis of a DNA-dependent RNA, possibly a m-RNA, and a particular type of proteins, possibly a specific enzyme, is needed for theylene to induce abscission. Reactions of this type could take place in a matter of a few hours and complete destruction of r-RNA and other polymeric nucleic acids could follow. Such destruction of r-RNA and complete disappearance of ribosomal particles have been reported (20, 23, 90, 114, 151, 152, 161, 164).

As to the effect of a 24-hour ethylene treatment on P^{32} incorporation into nucleic acids in fully expanded and mature leaves of calamondin, it was shown that the yield of phenol-extracted nucleic acids declined slightly. It was also found that there was no substantial change in s-RNA and r-RNA. However, the rate of incorporation of P^{32} into the DNA-RNA fraction was increased by more than 12-fold. It is widely accepted that the DNA-RNA complex is an intermediate in the transcription of genetic information (22, 63). Therefore, it is possible that this tremendous stimulation of DNA-RNA synthesis in calamondin leaves (Table 7) under the influence of ethylene for a short time is one of the basic reactions of ethylene. This concept is supported by the findings of Abeles (1) and Abeles and Holmes (2). It is possible that the action of ethylene may be exerted on certain genes which stimulate the synthesis of specific m-RNA that in turn initiate

the synthesis of degradative enzymes. These enzymes would then bring about the physiological and biochemical changes induced by ethylene.

SUMMARY AND CONCLUSIONS

From a study of the changes of phenol-extractable polymeric nucleic acids during natural growth and development and chemically modified development of citrus fruits and leaves with GA and ethylene, it is concluded that the loss of r-RNA in aging fruits and leaves could bring about the impairment of protein synthesis. This was thought to be due to the loss of ribosomal sites necessary for the polymerization of amino acids into protein. Ethylene may enhance the senescence process in citrus fruits and leaves by initiating reactions that would bring about the breakdown of r-RNA necessary for protein synthesis.

Also, it is concluded that GA retards senescence of citrus fruits by preventing the destruction of chlorophyll. This action may be through maintaining the integrity of the DNA and RNA systems of the chloroplast. This may also indicate a preferential action of GA on the nucleic acid systems of the chloroplast.

These conclusions are based on the following observations: Mature-green calamondin fruits were treated with ethylene and GA, and fully expanded leaves were treated with only ethylene to modify senescence, and the changes in chlorophyll content and various types of P^{32}-labeled polymeric nucleic acids were determined.

During the course of growth and development of calamondin fruits, there was an increase in s-RNA and a decrease in r-RNA with age. These changes were more pronounced in fruits that had attained maximum size

and chlorophyll was undergoing degradation. Developing leaves of calamondin showed also an increase in s-RNA as they aged; however, the magnitude of these changes are less drastic in leaves than in fruits. The DNA-RNA complex, considered as an intermediate in the transcription of genetic information, was extracted from young, fully expanded and senescent leaves. This fraction was extracted only from very young fruits (0.5-1.0 cm in diameter) and here it was in very small quantities.

Ethylene-treated, fully-grown calamondin fruits showed a slight increase in s-RNA and a slight decrease in r-RNA 1 day after the treatment. After 9 days, however, when the ethylene-treated fruits had lost most of its chlorophyll, no r-RNA was detected in the rind. In the meantime, nucleic acid extracts from non-treated fruits contained more than 40% r-RNA. When both ethylene-treated and non-treated fruits lost most of their chlorophyll after 28 days, their nucleic acid extracts showed no r-RNA, but only s-RNA. This pattern was confirmed by both absorbancy and radioactive phosphorus incorporation studies.

Ethylene treatment of healthy, mature leaves of calamondin resulted in about a 12-fold increase in the amount of P^{32}-labeled DNA-RNA which may indicate an increase in the synthesis of m-RNA.

GA was found to delay senescence of mature green 'Parson Brown' and 'Valencia' orange fruits as indicated by chlorophyll analysis and reflectance measurements. GA-treated 'Valencia' orange fruits stored in the dark for 5 weeks maintained a higher chlorophyll content than those which were not treated. In a concurrent test, GA did not bring about regreening of fully-colored 'Parson Brown' or 'Pineapple' orange fruits. When various concentrations of GA, ranging between

0 and 1,000 ppm, were tested on mature green 'Valencia' fruits, there was no detectable difference in color between fruits treated with different concentrations, however, there was a difference between the treated and control.

GA was also shown to maintain a higher level of r-RNA in the rind of mature-green calamondin fruits up to 14 days after the treatment. However, after 28 days, both the GA-treated and control fruits showed no r-RNA in their nucleic acid extracts despite the fact that senescence of treated fruits was delayed as indicated by a higher chlorophyll content.

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This dissertation was prepared under the direction of the Chairman of the candidate's Supervisory Committee and has been approved by all members of the committee. It was submitted to the Dean of the College of Agriculture and the Graduate Council and was prepared as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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